Sex-Specific Effects of *Cacna1c* Haploinsufficiency on Social Behavior, Ultrasonic Communication, and Cognition in Rats

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Theresa Marie Kisko

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Erstgutachter: Dr. Markus Wöhr, Philipps-Universität Marburg

Zweitgutachter: Prof. Dr. Hanna Christiansen, Philipps-Universität Marburg

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Summary

The risk gene *CACNA1C* encodes for the α1C subunit of the L-type voltage-gated Ca^{2+} channel, known as $Ca_v1.2$. Genome wide association studies have implicated *CACNA1C* in neuropsychiatric disorders, such as major depression, bipolar disorder, schizophrenia, and autism spectrum disorder. Importantly, social behavior and communication deficits are persistent in each disorder with added cognitive impairments similarly being present. Several studies in humans and mouse models have indicated that $Ca_v1.2$ expression levels are associated with alterations in sociability and cognition. Rat models provide an ideal translational tool to determine underlying disease pathomechanisms, due in part to their highly gregarious nature emerging early in life, thus, creating a practical means to study the development of social behavior and communication. Using a newly developed *Cacna1c* rat model, this dissertation aimed at exploring the role *Cacna1c* plays in social behavior and communication in juvenile rats, as well as the association with cognitive impairments in adulthood. Detailed practical assessment for juvenile behavior and ultrasonic vocalizations (USV) outline social play (Review I) and USV playback (Review II) as pertinent paradigms to assess alterations in social behavior and communication with relevance to neuropsychiatric disorders. Results indicate that deficits in 50-kHz USV were evident in male haploinsufficient *Cacna1c* rats in the sender and receiver (Study I). *Cacna1c* haploinsufficiency in females resulted in abnormal social play behaviour and minor deficits in response to 50-kHz USV playback (Study II). Moreover, *Cacna1c* rats appear to show normal, and in some cases above normal, cognitive abilities, albeit with a slight reduction in cognitive flexibility in haploinsufficient *Cacna1c* males (Study III). Together, these findings further extend the notion that $Ca_v1.2$ expression levels may be associated with alterations in social behavior, communication, and cognitive abilities in a sex-dependent manner, with important bearings on neuropsychiatric disorders.

Zussammenfassung*

Das Risikogen *CACNA1C* codiert für die α1C Untereinheit des spannungsabhängigen L-Typ-Calciumkanals $Ca_v1.2$. Genomweite Assoziationsstudien konnten einen Zusammenhang von *CACNA1C* mit neuropsychiatrischen Störungen, wie schwerer Depression, bipolarer Störung, Schizophrenie und Autismus-Spektrum-Störung, herstellen. Bedeutsamerweise gehen diese Störungen mit Defiziten im Sozial- und Kommunikationsverhalten sowie kognitiven Beeinträchtigungen einher. Mehrere Human- und Mausstudien haben bereits Hinweise darauf ergeben, dass $Ca_v1.2$-Expressionslevel mit Veränderungen der Soziabilität und Kognition einhergehen. Rattenmodelle bieten einen optimalen translationalen Forschungsansatz, um grundlegende Pathomechanismen zu bestimmen. Ratten bieten wegen ihrer geselligen Natur, die sich früh in der Lebensspanne ausbildet, besonders gute Möglichkeiten, um die Entwicklung von Sozial- und Kommunikationsverhalten zu untersuchen. Ziel dieser Doktorarbeit war es anhand eines neu entwickelten *Cacna1c*-Rattenmodells, den Einfluss von *Cacna1c* auf Sozial- und Kommunikationsverhalten von juvenilen Ratten und kognitive Beeinträchtigungen von adulten Ratten zu untersuchen. Eine detaillierte Analyse häufig verwendeter Methoden zur Erfassung juveniler Verhaltensweisen und Ultraschallvokalisationen (USV) zeigte, dass soziales Spielverhalten (Review I) und die Präsentation von 50-kHz USV (Review II) relevante Paradigmen zur Erfassung von Veränderungen des Sozial- und Kommunikationsverhaltens sind. Die erhobenen Befunde es zeigten sich Defizite in der Emission und Verarbeitung von 50-kHz USV bei männlichen haploininsuffizienten *Cacna1c* Ratten (Studie I). Eine *Cacna1c* Haploinsuffizienz bei weiblichen *Cacna1c* Ratten führte zu abnormalen sozialen Spielverhalten und geringfügigen Defiziten in Reaktion auf die Präsentation von 50-kHz USV (Studie II). Zudem wiesen *Cacna1c* Ratten normale beziehungsweise in Einzelfällen leicht erhöhte kognitive Fähigkeiten auf, wenngleich bei männlichen haploininsuffizienten *Cacna1c* Ratten eine leichte Verringerung der kognitiven Flexibilität festzustellen war (Studie III). Diese Arbeit erweitert bisherige Befunde, wonach $Ca_v1.2$-Expressionslevel mit Veränderungen des Sozialverhaltens, der Kommunikation und kognitiver Fähigkeiten in einer geschlechts-abhängigen Weise assoziiert sind, was wichtige Implikationen hinsichtlich neuropsychiatrischer Störungen hat.

* My sincere thanks to Tobias Redecker for his time and efforts in the translation of my summary to German.

Neuropsychiatric Disorders and *CACNA1C*

A high rate of heritability characterizes several major neuropsychiatric disorders (Sullivan. Daly, O'Donovan, 2012), for example the individual heritability of major depression is reported to be as high as about 40-50 % (Kendler & Prescott, 1999; Sullivan, Neale, & Kendler, 2000) bipolar disorder 60-85% (McGuffin, Rijsdijk, Andrew, et al., 2003; Smoller & Finn, 2003), and schizophrenia at rates of 60-85 % (Lichtenstein, Yip, Björk, et al., 2009). Moreover, recent heritability rates of autism spectrum disorder have been reported to be between 70-80 % (Bailey, Le Couteur, Gottesman, et al., 1995; Rosenberg, Law, Yenokyan, et al., 2009). High rates of heritability are a strong indication that genetic factors are playing a central role in these disorders (Craddock & Sklar, 2013; Escudero & Johnstone, 2014; Geschwind, 2011; Kessler, Chiu, Demler, & Walters, 2005; Lohoff, 2010). Over the years studies investigating genetic components for neuropsychiatric disorders have identified and established hundreds of risk genes (Consortium, 2013), with the cross-disorder risk gene *CACNA1C* being strongly implicated and reportedly replicated in bipolar disorder, major depressive disorder, schizophrenia and autism spectrum disorder. *CACNA1C* has recently emerged as a prime candidate susceptibility gene for neuropsychiatric disorders (Bhat, Dao, Terrillion, et al., 2012; Heyes, Pratt, Rees, et al., 2015; Ou, Crane, MacIntosh, et al., 2015), particularly because single-nucleotide polymorphisms (SNPs) in *CACNA1C* rank among the most consistent and replicable genetic findings from genome-wide association studies (GWAS) (Liu, Blackwood, Caesar, et al., 2011; Schizophrenia Psychiatric Genome-Wide Association Study (GWAS) Consortium, 2011; Wray, Pergadia, Blackwood, et al., 2012).

CACNA1C

The gene *CACNA1C* encodes for the α1C subunit of the L-type voltage-gated Ca^{2+} channel (LTCC), known as $Ca_v1.2$. Along with $Ca_v1.2$, the LTCC family includes three other distinct members known as $Ca_v1.1$, $Ca_v1.3$, and Ca_v 1.4 (Catterall, 2011). $Ca_v1.2$ regulates depolarization-dependent Ca^{2+} influx into the cell (Sinnegger-Brauns, Huber, Koschak, et al., 2009) and has an important role in modulating neuronal excitability, synaptic plasticity and gene expression, which has been experimentally demonstrated in rodent studies (Zamponi, 2016; Zuccotti, Clementi, Reinbothe, et al., 2011). In the mammalian brain, the main LTCC

expressed is $Ca_v1.2$. Quantitative polymerase chain reactions (qPCR) of RNA transcripts in the mouse brain have shown that $Ca_v1.2$ totals almost 85% of all LTCCs with $Ca_v1.3$ accounting for the majority of the remainder (Sinnegger-Brauns et al., 2009). In $Ca_v1.2$ channels there are three subunits consisting of: transmembrane α1C (*CACNA1C*), $\alpha_2\delta$ (encoded by *CACNA2D-1, 2, or 3*), intracellular β (encoded by *CACNB1-4* genes) as well as calmodulin (CaM) (Dolphin, 2009). Whereas, auxiliary *CACNA2D-1-3, CACNB1-4* and CaM are all involved in regulation of expression and modulating select properties of $Ca_v1.2$, C*ACNA1C*, specifically, encodes for several major characteristics of the $Ca_v1.2$ channel including: voltage-sensing, ion selectivity, and pharmacological responses associated with the binding of Ca^{2+} channel blockers (Bhat et al., 2012).

Disorders Linked to *CACNA1C*

Amongst others, four major neuropsychiatric disorders have been strongly linked to *CACNA1C.* This includes the affective disorders: Major depressive disorder (MDD; (Dao, Mahon, Cai, et al., 2010; E. K. Green, Grozeva, Jones, et al., 2010)) and Bipolar disorder (BPD; (Ferreira, O'Donovan, Meng, et al., 2008; Moskvina, Craddock, Holmans, et al., 2009; Sklar, Ripke, Scott, et al., 2011; Sklar, Smoller, Fan, et al., 2008)) as well as the neurodevelopmental disorders: Schizophrenia (SCZ; (E. K. Green et al., 2010; Nyegaard, Demontis, Foldager, et al., 2010; Ripke, Neale, Corvin, et al., 2014; Schizophrenia Psychiatric Genome-Wide Association Study (GWAS) Consortium, 2011)) and Autism spectrum disorder (ASD; (D'Gama, Pochareddy, Li, et al., 2015; Li, Zhao, You, et al., 2015; Splawski, Timothy, Decher, et al., 2005; Splawski, Timothy, Sharpe, et al., 2004)). Furthermore, it has been repeatedly shown that mutations in *CACNA1C* resulting in a gain of function, causes Timothy syndrome (TS), which is a developmental disorder in which the phenotypic range includes ASD (Liao & Soong, 2010; Splawski et al., 2004).

Further support for the role *CACNA1C* in MDD, BPD, and SCZ, comes from clinical studies linking the primary *CACNA1C* risk allele associated with neuropsychiatric disorder, rs1006737, to alterations in brain structure and function, both in patients (Backes, Dietsche, Nagels, et al., 2014; Mallas, Carletti, Chaddock, et al., 2016; Soeiro-de-Souza, Bio, Dias, et al., 2013; Zhang, Shen, Xu, et al., 2011) and healthy individuals (Erk, Meyer-Lindenberg, Schnell, et al., 2010; Krug, Nieratschker, Markov, et al., 2010; Paulus, Bedenbender, Krach, et

al., 2014; Strohmaier, Amelang, Hothorn, et al., 2013; Wessa, Linke, Witt, et al., 2010). For example, rs1006737 is associated with variations within the prefrontal cortex (PFC)-amygdala-hippocampus circuit structure (Dietsche, Backes, Laneri, et al., 2014; Perrier, Pompei, Ruberto, et al., 2011; Wang, Mcintosh, He, Gelernter, & Blumberg, 2011) and function (Bigos, Mattay, Callicott, et al., 2010; Dima, Jogia, Collier, et al., 2013; Jogia, Ruberto, Lelli-Chiesa, et al., 2011; Tesli, Skatun, Ousdal, et al., 2013), which has been suggested to be the underlying factor modulating the anxiety and depression intermediate phenotypes commonly observed in MDD, BPD, and SCZ (Erk et al., 2010).

Behavioral effects supporting the impact of the *CACNA1C* risk allele on the PFC-amygdala-hippocampus circuit have shown reduced affective startle responses to pleasant pictures and exaggerated responses to negative pictures in healthy *CACNA1C* rs1006737 risk allele carriers (Pasparakis, Koiliari, Zouraraki, et al., 2015). Associations with low extraversion in *CACNA1C* rs1006737 risk allele carriers (Roussos, Giakoumaki, Georgakopoulos, Robakis, & Bitsios, 2011) and functional alterations in social behavior during a facial affect processing task have also been reported, showing an increased activation of the fusiform gyrus, which mediates facial perception (Dima et al., 2013). This suggests that there may also be a dysregulation in neural circuitry required for facial processing in social interaction. Specifically, the ventral anterior cingulate cortex (vACC) has recently been implicated in healthy rs1006737 risk allele carriers with childhood interpersonal trauma in response to emotional valence of various faces. In response to angry compared to happy faces, the vACC showed a stronger deactivation. Limbic structures encoded by *CACNA1C* and implicated in neuropsychiatric disorders provide a look into how the regulation of LTCCs within these structures may influence the manifestation of the disorders. For example, the hypothalamic-pituitary-adrenal (HPA) axis is regulated by increased Ca^{2+} currents mediated by glucocorticoids (Chameau, Qin, Spijker, Smit, & Joëls, 2007; Karst, Nair, Velzing, et al., 2002), and mood disorders, such as MDD and BPD, have been strongly linked to HPA axis reactivity (Gunnar, Frenn, Wewerka, & Van Ryzin, 2009).

In healthy *CACNA1C* rs1006737 risk allele carriers the data available on the influence of the *CACNA1C* gene on cognitive function, is conflicting. Some studies report in healthy *CACNA1C* rs1006737 risk allele carriers there is lower verbal fluency (Krug et al., 2010), attentional deficits (Thimm, Kircher, Kellermann, et al., 2010), impaired working memory

(Zhang et al., 2011), and poorer learning performance (Dietsche et al., 2014). In contrast, several studies report no association between the gene and verbal learning and memory, verbal intelligence (Erk, Meyer-Lindenberg, Linden, et al., 2014; Erk et al., 2010; Roussos et al., 2011), working memory (Paulus et al., 2014), recognition memory (Dietsche et al., 2014), or overall cognitive functioning (Hori, Shimoju, Tokunaga, et al., 2013; Soeiro-de-Souza et al., 2013). In terms of structural and functional alterations during cognitive tasks there is, again, somewhat contradicting evidence of increased activity in the PFC (Bigos et al., 2010) as well as reduced activation (Erk, Meyer-Lindenberg, Linden, et al., 2014; Erk et al., 2010; Krug, Witt, Backes, et al., 2014; Paulus et al., 2014). However, during a semantic verbal fluency task, increased activation in regions of the frontal gyrus have been found in both healthy rs1006737 risk allele carriers (Krug et al, 2010) and in risk allele carriers suffering from depression (Backes et al., 2014).

Importantly, the disorders above for which *CACNA1C* appears to have a role all have a significant worldwide prevalence. Rates of people affected are reported for MDD, BPD, SCZ and ASD to be as high as 300 million, 60 million, 21 million and, 1 in 68, respectively (Christensen, Grønborg, Sørensen, et al., 2013; "WHO | Autism spectrum disorders," 2017, "WHO | Mental Disorders," 2017). With global prevalence rates being so high and impacting so many people the need for investigation into the pathophysiology of these disorders is crucial. Each of the four disorders share common characteristics and impact the processing of reward, mood and emotion and cognition. Most notably, however, one important characteristic that is severely impacted in MDD, BPD, SCZ, and ASD is social functioning and cognition (American Psychiatric Association, 2013). Humans are a naturally gregarious species and since the beginning of human evolution social functioning as well as cognitive abilities have been important components working together to benefit our survival. Therefore, with so many people worldwide suffering from affective and neurodevelopmental disorders in which social functions and variation in cognitive domains are drastically impaired, and in extreme cases even results in suicide, it seems essential for research focus to be placed on the causes and potential repair of these deficits.

Major Depressive Disorder

MDD is a common and severe mood disorder and is suggested to be the leading cause of disability worldwide and contributes significantly to the overall global burden of disease ("WHO | Depression," 2017) with more women than men appearing to suffer from MDD (Richards, 2011). There is a strong link to genetic influence with some reports being as high as 40-50% heritability (for review see: (Sullivan et al., 2000). MDD is characterized by anhedonia; meaning, the inability to feel pleasure in normally pleasurable activities, and negative moods which are persistent across situations. Often, MDD patients experience low self-esteem, a loss of interest in normally enjoyable activities, low energy and pain without any cause. Evidence from controlled clinical trials, as well as, follow-up studies show that impairments in social functioning in depressed patients are significant, pervasive and persistent (Hirschfeld, Montgomery, Keller, et al., 2000). In two studies conducted by Erk et al (2014; 2010) the authors found that associations between *CACNA1C* and structural and functional alterations within the PFC–amygdala–hippocampus circuit resulted in negative correlations between regional activation in the hippocampus and depression and anxiety scores. Patients with MDD can sometimes experience cognitive impairments, most notably, in the areas of attention, executive function and memory and these appear to be present even when the individuals are euthymic, meaning in the normal range or mood; neither depressed nor elevated (Hammar & Årdal, 2009). For example, depressed patients with the *CACNA1C* rs1006737 risk allele show alterations in structure and functioning of frontal gyrus areas during a semantic verbal fluency task (Backes et al., 2014).

Bipolar Disorder

BPD is characterized by episodic recurrent pathological mood disturbances and has no reliable biological markers and therefore is described by its clinical features outlined in the diagnostic and statistical manual of mental disorders (DSM-5) (American Psychiatric Association, 2013) as well as in the international statistical classification of diseases and related health problems (ICD-10) (World Health Organization, 1992). The range of mood disturbances seen in BPD patients can be from extreme elation (mania), to severe depression. Often, BPD can be accompanied by psychotic features, such as delusions and hallucinations, as well as, disturbances in thinking and behavior. Over the years of research into the

pathophysiology of BPD, it has been found that genetics appear to play a significant role in affecting whether an individual will be predisposed to the disorder or certain phenotypes of the disorder (Smoller & Finn, 2003). Social impairments are concomitant to affective disorders (for comprehensive review see: (Sanchez-Moreno, Martinez-Aran, Tabarés-Seisdedos, et al., 2009), however, they appear to be slightly more marked in BPD. According to Sanchez-Moreno et al (2009) after the remission of manic or depressive episodes, a recovery of social functioning is expected. However, BPD patients often still report deficits in social functioning, and even in remission people with BPD tend to have fewer social interactions with friends (Sanchez-Moreno et al., 2009). Reports show that this deficit can be sustained as long as 2 years following the initial onset of the disorder (Sanchez-Moreno et al., 2009), but may also persist for up to 10 years (Goldberg & Harrow, 2004). Likewise, poor social functioning may lead to more depressive symptoms and has even been reported by Gitlin et al (1995) to predict shorter times to relapse. Of note, associations between *CACNA1C* and bipolar disorder patients has shown impairments in activation and connectivity during emotional (Radua, Surguladze, Marshall, et al., 2013; Tesli et al., 2013) and facial affect processing (Dima et al., 2013; Soeiro-de-Souza et al., 2013). Along with emotional and social impairments, cognitive deficits have also been reported in BPD patients, with nearly 40% of early onset patients showing cognitive impairments in later life (Tsai, Lee, Chen, & Huang, 2007). Long-term difficulty in remembering names and conversations in the past appears to be a common complaint from patients with BPD (Sanchez-Moreno et al., 2009) and in effect, patients with lower psychosocial functioning show more generalized verbal memory deficits. The *CACNA1C* rs1006737 risk allele has also been implicated in executive function (Arts, Simons, & Os, 2013; Soeiro-de-Souza et al., 2013) and working memory (Zhang et al., 2011) in individuals suffering from BPD.

Schizophrenia

SCZ has a diverse psychopathology and is characterized by positive symptoms which include delusions, hallucinations, and psychotic symptoms typically manifesting as a loss with reality; negative symptoms such as, impaired motivation, reduction in spontaneous speech, social withdrawal (Owen, Sawa, & Mortensen, 2016)), and cognitive impairments (Joyce & Roiser, 2007). Similar to BPD, there are no reliable biological markers for SCZ and it is largely

described by its clinical features outlined in the DSM-5 (American Psychiatric Association, 2013). Within the core features of SCZ, the negative symptoms appear to be the most debilitating and typically are chronic and associated with long-lasting effects on social functioning (Owen et al., 2016). A common issue with SCZ, and studies investigating social impairments within, is that several of the negative symptoms can often result in social withdrawal (Harvey, Lombardi, Leibman, et al., 1996; McGurk & Meltzer, 2000) and therefore, it is not easy to distinguish between the persistent negative symptoms and impairments in social functioning (Burns & Patrick, 2007). Some have suggested that the cause of poor social functioning is in large part a result of deficits specifically within social cognition, i.e., emotion recognition, self-regulation and understanding the mental states of others (Dodell-Feder, Tully, Lincoln, & Hooker, 2014; Savla, Vella, Armstrong, Penn, & Twamley, 2013). SCZ is highly associated with dysfunction in neural areas including; amygdala, medial prefrontal cortex (mPFC), striatum, anterior cingulate cortex (ACC) and hippocampus, and it appears that the degree of dysfunction can be correlated to social cognitive performance and real-world social behavior (Dodell-Feder et al., 2014). Along with impairments in social cognition, individuals with SCZ also experience deficits in a variety of other cognitive domains, such as executive function, attention, memory and language (Kuperberg & Heckers, 2000). Numerous imaging studies have corroborated the key brain regions listed above as being associated with distinct patterns of dysfunction (Barch & Ceaser, 2012; Kuperberg & Heckers, 2000). Notably, recent studies investigating the *CACNA1C* rs1006737 risk allele in SCZ patients showed alterations in both working memory (Zhang et al., 2011) and executive function (Arts et al., 2013; Soeiro-de-Souza et al., 2013) resulting in cognitive deficits (Dietsche et al., 2014; Erk, Meyer-Lindenberg, Linden, et al., 2014; Krug et al., 2014; Paulus et al., 2014). In parallel with the cognitive deficits, poor social functioning can be predicted by cognitive deficits in SCZ (Kuperberg & Heckers, 2000), with verbal memory and vigilance deficits being the best predictors for social functioning impairments within a community (M. F. Green, 1996). Similar to MDD and BPD, SCZ can have moderate to severe episodes and has the potential to become a chronic and debilitating illness in which the individuals experience long-lasting effects on their social and cognitive functioning and at its worst can lead to suicide.

Autism Spectrum Disorder

In humans the main characteristics of ASD include the concomitant occurrence of impaired social interaction and communication as well as repetitive and stereotyped patterns of behavior (American Psychiatric Association, 2013). Corresponding, to the above-mentioned disorders of MDD, BPD and SCZ, reliable biological markers are not yet available to accurately diagnose persons with autism. Rather, the diagnosis is done mainly through behavioral observation following closely the outlined characteristics of ASD in the DSM-5 (American Psychiatric Association, 2013). It is typically characterised by early childhood onset with atypical development and exists with a 4:1 ratio in males over females (Lai, Lombardo, & Baron-Cohen, 2014). In contrast to MDD, BPD and SCZ, ASD represents a clinically variable population that suffers from pathological levels of widespread variability in major cognitive and behavioral realms that are disrupted, instead of a distinct clinical disorder (Geschwind, 2011). This is supported by the findings in numerous studies that both high and low IQ levels can be found in individuals with ASD, and that the disorder is typically associated with an uneven profile of cognitive abilities and language development (for review see: (Charman, Jones, Pickles, et al., 2011)). Recently, ASD has been associated with mutations in $Ca_v1.2$ channels. More specifically, mutations in the channel cause TS which results from an identical de novo G406R mutation in exon 8a or a G402S mutation in exon 8 of the *CACNA1C* gene (Splawski et al., 2005, 2004). Functional expression shows that the G406R mutation produces and maintains inward Ca^{2+} currents by causing a near complete loss of voltage-dependent channel inactivation (Splawski et al., 2004) in other words, it causes a gain of function. Multisystem dysfunction and developmental defects such as severe deficits of language and social development, characterize TS, which also meets criteria for ASD, suggesting the importance of Ca^{2+} signaling in human development. Furthermore, SHANK scaffolding proteins, strongly associated with ASD (Monteiro & Feng, 2017), have been implicated in the regulation of LTCCs, including $Ca_v1.2$ channels, and thus, mutations in the *SHANK* gene family could lead to malfunctions or irregularities in $Ca_v1.2$ (Pym, Sasidharan, Thompson-Peer, et al., 2017), possibly contributing to ASD-related phenotypes.

CACNA1C and Disease Mechanisms

While the *CACNA1C* mutation associated with ASD is a missense mutation, G402S or G406R in exons 8 or 8a, and results in a gain of function (Splawski et al., 2005, 2004), the consequences of *CACNA1C* mutations in BPD, MDD and SCZ are less clear. This is because given the location of SNPs, such as rs1006737, being on the non-coding region of the gene they are not expected to interfere with the structural-functional properties of the $Ca_v1.2$ channels, as is the case for G402S or G406R mutations in TS. However, as rs1006737 and other identified SNPs are found in the intronic, i.e. the non-protein coding, region of *CACNA1C*, neurobiological alterations whereby SNPs modify brain structure and function likely are dependent on changes in *CACNA1C* expression levels. Consistent with this view, the rs1006737 risk allele was found to be associated with enhanced *CACNA1C* mRNA expression in post-mortem tissue (Bigos, Mattay, Callicott, et al., 2010) and induced human neurons (Yoshimizu, Pan, Mungenast, et al., 2015), yet others reported decreased *CACNA1C* expression levels in the brains of SCZ (Roussos, Mitchell, Voloudakis, et al., 2014) and BPD (Gershon, Grennan, Busnello, et al., 2014) patients carrying the rs1006737 risk allele, suggesting that both increased and decreased expression might be associated with neuropsychiatric disorders in humans. Little is known about the direct role $Ca_v1.2$ expression levels have on behavioral phenotypes characteristic of MDD, BPD, SCZ and therefore, investigations using animal models in which expression levels can be manipulated for example, are important to gain a better idea about the influence of decreased expression levels on subsequent social and cognitive behavioral domains and how this may impact the presence of neuropsychiatric phenotypes.

Animal Models for Neuropsychiatric Disorders

Animal models, specifically rodents, often prove a useful tool for uncovering mechanisms underlying characteristic behavioral phenotypes, likely, as a result of the overlapping emotional and behavioral circuitry (Cenci, Whishaw, & Schallert, 2002; Cryan & Holmes, 2005; van der Staay, Arndt, & Nordquist, 2009). Some of the most commonly used models include mice and rats (Cenci et al., 2002; Cryan & Holmes, 2005; Silverman, Yang, Lord, & Crawley, 2010; Weiss, Lightowler, Stanhope, Kennett, & Dourish, 2000; Wöhr & Scattoni, 2013).

In modeling neuropsychiatric disorders, mice have fast become the preferred animal choice when looking into relevant characteristics and phenotypes , and the resulting effects of gene manipulation (Cryan & Holmes, 2005). For example, behavioral symptoms and morphological abnormalities similar to those seen in SCZ can be modeled by mutating the susceptibility gene DISC1 in mouse genetic material (Pletnikov, Ayhan, Nikolskaia, et al., 2008). The Rouen 'depressed' mice are also one of the most encouraging and relevant depression models, as these mice have a high occurrence of depressive behavioral phenotypes, increased levels of corticosterone and serotonergic dysfunction which is highly associated with depression (Cryan & Mombereau, 2004). In contrast to MDD mouse models, BPD models involving a genetic mutation are less common, as the identification of genetic risk factors is still in the emerging stages (Craddock & Sklar, 2013). However, recently GSK-3β transgenic mice have been developed, by overexpressing glycogen synthase kinase-3β, as a model for hyperactivity and mania was with hopeful translational relevance (Prickaerts, Moechars, Cryns, et al., 2006). Furthermore, modelling SHANK mutations in transgenic mice has become a potentially promising technique to determine mechanisms, causes, treatments and the overall etiology of ASD (Sungur, Schwarting, & Wöhr, 2017; Yoo, Bakes, Bradley, Collingridge, & Kaang, 2014).

When studying rodent models, typically, there are several standard behavioral paradigms employed which allow the researchers to assess several aspects at once in an easy, practical and adaptable manner. For example two of the most common paradigms include, the open-field, light-dark test and elevated-plus maze (EPM) test (Cryan & Holmes, 2005; Weiss et al., 2000). Spontaneous behavior in an open field can be used to test general psychomotor function and exploratory behavior, but also anxiety or inversely risk taking by means of spending more time in the centre compared to the edges (Cryan & Holmes, 2005; Weiss et al., 2000). A variation of the open field, the light-dark test, in which one side is painted dark and the second side is brightly illuminated, assumes that the animals will avoid the bright side, thus, reducing locomotor behavior and inducing anxiety (Weiss et al., 2000). The EPM uses the same principles of open field and the light-dark test and measures, exploratory behavior, anxiety, as well as risk taking behavior by means of the percentage of arm entries and the amount of time spent on open arms (Weiss et al., 2000).

Other standard models, typically used to assess depression–like, or antidepressant behaviors consist of, the Porsolt forced swim test (FST) (Can, Dao, Arad, et al., 2012; Porsolt, Bertin, & Jalfre, 1977), and tail suspension test (TST) (Bergner, Smolinsky, Hart, et al., 2010). During the FST emotional despair is quantified by floating behavior in response to being placed into a tank of water in which the animal has no ability to touch the bottom of the tank with their feet while their head is above the water (Porsolt et al., 1977). Along the same lines as FST, but not synonymous, is the TST in which mice hang upside-down by their tail and passive immobility, after a few minutes of futile struggling, is quantified (Bergner et al., 2010). By adapting the standard assays mentioned above i.e., open-field and EPM, endophenotypes of disorders such as BPD can be assessed. For instance, modeling mania in animal models is most often done by treating normal control animals with psychostimulants, such as cocaine but more commonly with amphetamine and then measuring hyperactivity or hyper-locomotion in one of the standard paradigms, for example open-field (Young, Henry, & Geyer, 2011). By repeatedly administering psychostimulants sensitization can occur in which case a further injection with treatment such as, lithium or valproate, commonly used in human patients, would be expected to blunt the effect. In which case the comparison of hyperactivity and hyper-locomotion before and after treatment is typically assessed (Young et al., 2011).

Aside from measures of anxiety and depression, another key paradigm for studying emotional changes, central to psychiatric disorders, is fear conditioning. Using an aversive stimulus, such as a foot shock (unconditioned stimulus, US), repeatedly paired with a formerly neutral stimulus, such as a tone (conditioned stimulus, CS) the freezing responses; complete lack of somatic mobility aside from breathing (Fendt & Fanselow, 1999), and recently 22-kHz ultrasonic vocalizations (Wöhr, Borta, & Schwarting, 2005), are measured as indices of fear learning. The CS typically gains the efficacy to elicit fear related responses in the absence of the US.

Cognitive measurement tasks such as spatial learning paradigms, including Morris water maze (MWM) (Morris, 1981) and radial arm maze (Olton & Samuelson, 1976), provide a translational method for investigating human declarative abilities (Morellini, 2013) which are often diminished in neuropsychiatric disorders (Austin, Mitchell, & Goodwin, 2001; Cirillo & Seidman, 2003; van Gorp, Altshuler, Theberge, & Mintz, 1999). The MWM test places animals in a large circular maze filled with opaque water and in order to escape the water the animals

must learn based on spatial cues, the location of an invisible, slightly submerged platform. The latency to locate the platform and length of swimming paths are measured over numerous trials, with the expectation that more trial leads to shorter latencies and swimming paths (Morellini, 2013). The radial maze test typically uses food-deprived rats, rather than mice, and encourages them to learn the locations of food rewards placed at the end of specific arms of the radial maze (Morellini, 2013). The ability to test spatial as well as working memory is a benefit of this test as opposed to the MWM. The number of correct arm entries i.e., arms with food reward, and the number of incorrect arm entries, i.e., arms without food or ones already visited are typically quantified and compared (Morellini, 2013).

Developed in the last decade, testing social deficits with relevance to ASD and other neuropsychiatric disorders can be measured through a comprehensive set of behavioral assays for detecting impairments in social interaction and communication (Silverman et al., 2010; Wöhr & Scattoni, 2013). For instance, social behaviors in mice are typically assessed using the three-chambered social approach assay, with intact sociability being defined as spending more time in proximity to a conspecific over an empty corral (Silverman et al., 2010). Measuring communication deficits in ASD mouse models can be difficult, as it is not yet well understood. Several different modalities such as ultrasonic vocalizations, visual and gustatory cues as well as tactile information may all contribute to information communication and social bonding (Silverman et al., 2010). A few different paradigms are typically employed to test olfactory cues derived from conspecifics and the subsequent response to the cues by other mice. One common assay, for example, is the olfactory habituation/dishabituation test. In this test the animal is presented with several odours over a period of time and the idea is, that the novel odours, especially the social odours, will elicit more sniffing time than any repeated exposure to already presented odours such as a banana scent, for example (Silverman et al., 2010). Repetitive behavior, in which sequences of persistent self-grooming, marble burying or repetitive digging, in addition to stereotyped behaviours, i.e., circling, jumping, backflips and self-grooming, are also commonly measured in ASD animal models by simply scoring the behaviors using a pre-defined score analysis (Silverman et al., 2010). The repetitive and stereotyped behaviour test examines two common representative behavioral traits observed in individuals with ASD and outlined in the DSM-5 (American Psychiatric Association, 2013).

Cacna1c Mouse Models

Rodent models, particularly mouse models, for *Cacna1c* have corroborated several of the findings seen in human *CACNA1C* data linking anxiety and anti-depressive behavioral endophenotypes associated with neuropsychiatric disorders (Dao et al., 2010). Translational relevance provided by mouse models does provide some insight as to the role of *Cacna1c* in the neurocircuitry of psychiatric disorders.

There is a strong relationship to changes in mood and emotion for people who suffer from depression and anxiety and studies investigating the role for $Ca_v1.2$ (Dao et al., 2010; Lee, Ra, Rajadhyaksha, et al., 2012) in mice have begun to dissect the underlying neurocircuitry involved, for example, within the PFC-amygdala-hippocampus circuit (for review see: (Kabir, Lee, & Rajadhyaksha, 2016)). Using heterozygous *Cacna1c* knockout mice in which *Cacna1c* expression is globally reduced, it has been determined that both the females (Dao et al., 2010) and males (Bader, Faizi, Kim, et al., 2011) exhibit increased levels of anxiety-related behavior. More targeted knockouts, focusing on forebrain *Cacna1c* expression, have confirmed the anxiety phenotype, suggesting that the *Cacna1c* levels in forebrain areas are particularly important in the regulation of anxiety (Lee et al., 2012). The above mouse models created by Dao et al (2010) and Lee et al (2012) both have a loss of function in $Ca_v1.2$, however, there is evidence suggesting that a gain of function with relevance to TS can also be detrimental to $Ca_v1.2$ channels. In human post-mortem brain tissue both an increase and decrease of *CACNA1C* mRNA levels have also been observed (Bigos et al., 2010; Gershon et al., 2014; Roussos et al., 2014; Yoshimizu et al., 2015). Bader et al (2011) explored a gain of *Cacna1c* function using a knockin $Ca_v1.2$ TS model and found, in contrast to knockouts, that there were no alterations in anxiety levels, indicating that it is the loss of function that is likely driving anxiety-like phenotypes. In contrast to anxiety-like phenotypes, studying the role of *Cacna1c* in depression has not provided much insight as of yet, however Dao et al (2010) have published findings, using the *Cacna1c* heterozygous mice, that shows an anti-depressant phenotype during FST and TST, which has been recapitulated in conditional PFC *Cacna1c* knockout mice (Kabir, Che, Fischer, et al., 2017). Evidence from *Cacna1c* mouse models have provided some indication as to a similar relationship in terms of higher anxiety (Dao et al., 2010; Lee et al., 2012) seen in human rs1006737 risk allele carriers and patients and also in mice with alterations in the *Cacna1c* gene. A relationship between *Cacna1c* mice and humans in regards

to depressive phenotypes, however, is still minimal with just two studies finding anti-depressant phenotypes (Dao et al., 2010; Lee et al., 2012). Therefore, more research in this area is warranted in order to be able to fully discuss the role of *Cacna1c* in depression. Additionally, Dao et al (2010) showed that when administered amphetamine *Cacna1c* heterozygous females have an attenuated response, suggesting that there may be impairments in the dopaminergic system as a result of the decrease in $Ca_v1.2$ expression. This is further supported in a study by Terrillion et al (2017) in which they show that mice with only one functional copy of *Cacna1c* gene manifest a diminished locomotor response to psychostimulant administration indicating that *Cacna1c* modulates mesolimbic-dopamine-dependent behavior.

In terms of evaluating *Cacna1c* in emotional fear processing in mice, the results are unfortunately not particularly strong in either direction as to the specific role *Cacna1c* plays. There are some indications that *Cacna1c* impairs emotional fear learning (Bauer, Schafe, & LeDoux, 2002; Davis & Bauer, 2012; Jeon, Kim, Chetana, et al., 2010; Langwieser, Christel, Kleppisch, et al., 2010) but others that find no evidence for any impairments (Langwieser et al., 2010; McKinney, Sze, White, & Murphy, 2008). However, evidence for a role of increased $Ca_v1.2$ expression comes from Bader et al (2011) in which, TS $Ca_v1.2$ knockin mice show increased contextual as well as cue-associated fear memories that persisted for up to two weeks after conditioning.

While there may be a fair amount of research into cognitive deficits in human *CACNA1C* risk allele rs1006737 carriers there is a surprising lack of information available using animal models. To date, only a few mouse studies provide any indication as to a likely role for *Cacna1c* in cognition and learning. In conditional *Cacna1c* forebrain knockout mice the results indicated that there were long-term deficits in the recall of spatial memory, lasting up to 30 days (J. A. White, McKinney, John, et al., 2008) as well as signs of enhanced cell death in young hippocampal neurons (Lee, De Jesus-Cortes, Kabir, et al., 2016). Equally, Moosmang et al (2005) found that there were severe impairments in the spatial learning of hippocampus conditional *Cacna1c* knockouts. However, in contrast, *Cacna1c* knockin TS mice showed normal acquisition of learning and memory but showed severe deficits in reversal learning (Bader et al., 2011). Suggesting, that it is the decrease in expression and that results in cognitive deficits and not a gain of function. Additionally, Temme et al (2016) found that during learning

acquisition of the Morris water maze, hippocampus conditional *Cacna1c* knockout mice had no impairment, however, if the task was made more difficult, such as by removing some spatial cues, they were unable to form the memory for the task (Temme et al., 2016). Of note, expression of *Cacna1c* is present throughout the entire mouse brain, with particularly high expression in the hippocampus (Allen Institute for Brain Science, 2004). Within many neuropsychiatric disorders, cognitive deficits can be as crippling to an individual as emotional and mood impairments can be, which is why investigating cognitive deficits as a result of *Cacna1c* impairments is important in determining the neurocircuitry and molecular pathways involved, in hopes of creating better treatment methods. The *Cacna1c* mouse evidence, while limited, does shed some light on understanding the functional role of *Cacna1c* in cognitive defects, specifically those involving the hippocampus, similarly seen in neuropsychiatric patients.

Social behavior and communication impairments are core features seen in disorders such as, MDD, BPD, SCZ and, ASD. Using the classic three-chambered social approach assay, Kabir et al (2017) and Dedic et al (2017) found that male forebrain *Cacna1c* null mutant mice do not show a preference for the conspecific. A lack of sociability was also evident in PFC *Cacna1c* knockdown mice (Kabir et al., 2017) but not in nucleus accumbens (NAcc) (Terrillion, Francis, Puche, Lobo, & Gould, 2017). However, no evidence for social deficits were obtained in constitutive *Cacna1c* heterozygous mice (Dedic et al., 2017), with one study even reporting enhanced sociability in a newly developed social home cage assay (Bader et al., 2011). There is a noticeable lack of research into the role of *Cacna1c* on social behavior and communication with only the few studies mentioned above investigating the impairments as a result of *Cacna1c* expression levels. One potential reason for the lack of social investigation in *Cacna1c* mouse models is the fact that mice sociability is not as translational to humans as that of the rat.

Cacna1c Rat Model and the Advantages of Rats

Limitations in mouse models has led to the creation of a transgenic *Cacna1c* rat. Using a previously established protocol by Geurts et al (2009), *Cacna1c* heterozygous rats were generated through zinc finger nuclease technology at SAGE labs. Specifically, the heterozygous *Cacna1c* rats carry a four base-pair (bp) deletion at 460649-460652 bp in

genomic sequence resulting in an early stop codon in exon 6. Once generated, the mutation can be faithfully and effectively transmitted through the germline (Geurts et al., 2009). The process creates a reduction of approximately 50% in mRNA and protein levels which results in loss of the $Ca_v1.2$ subunit. Importantly, there is a global decrease in expression levels meaning that, homozygous *Cacna1c* rats are not able survive the embryonic stages.

The method of Zinc finger nuclease technology done by Geurts et al (2009) as well as other methods including N-ethyl-N-nitrosourea (ENU) mutagenesis (Zan, Haag, Chen, et al., 2003), homologous recombination (Tong, Huang, Ashton, Li, & Ying, 2011) and most recently, clustered regularly interspaced short palindromic repeats (CRISPR)/Cas-mediated genome editing (Shao, Guan, Wang, et al., 2014) has opened the door and further allowed researchers to use more rat models when pursuing translational methods for human disease representations. Arguably, rats have several advantages over mice, for example they are larger in size and weight, which provides benefits to certain surgical procedures, and rats are much easier to handle and are less stressed by human contact than are mice, which tend to become more stressed with repeated handlings (Ellenbroek & Youn, 2016; Homberg, Wöhr, & Alenina, 2017). Moreover, rats appear to be more adept, meaning less affected by stress or thigmotaxis, than mice and rats tend to perform more stably over time in cognitive tasks (Ellenbroek & Youn, 2016). The principal difference, however, between rats and mice is observed in social behavior and social communication systems, in which case the rat appears to be far more pragmatic (Ellenbroek & Youn, 2016; Homberg et al., 2017; Pellis & Pellis, 2009). Rats are a much more gregarious species than mice and tend to live in large groups associations (Whishaw & Kolb, 2009) with a clear dominance hierarchy (Baenninger, 1966), and crucial role for social interactions beginning early in development, possibly even several days after birth (Meaney & Stewart, 1981). Additionally, it has been shown that when eating, rats prefer to do so in the proximity of their counterparts rather than alone (S. A. Barnett & Spencer, 1951). Therefore, this intense role for peers and social interactions in the brain and behavioral development of rats makes them an ideal candidate to study the emergence, progression, and mechanisms behind social behavior, functioning and communication and the resulting impairments seen in disorders such as in MDD, BPD, SCZ and ASD.

Similar to humans, rats have a critical period of development in which their brain develops and learns how to cope with their environmental and social surroundings (Opendak, Gould, &

Sullivan, 2017). During this critical period in a rat pup's life, conspecific interactions are indispensable for adequate development of social responses in adulthood (Van Den Berg, Hol, Van Ree, et al., 1999). A frequently used behavioral paradigm, termed juvenile social isolation, investigates the effects of social deprivation in juvenile rats during this critical brain developing period, by isolating them directly after weaning from both the mother and their littermates. Evidence has shown repeatedly that a prolonged lack of social interaction in the post-weaning period can lead to prominent social impairments in adulthood (Pellis & Pellis, 2009; Van Den Berg, Hol, et al., 1999). Thus, the post-weaning period is considered by many to be the critical period for social development in rats and coincides with the time when primary social bonds are formed (Scott & Marston, 1950). During this time of a juvenile rat's life, they spend increasing amounts of time away from the mother and their nests and begin to exhibit the first-non-maternal directed social interactions at littermates and other conspecifics. These early peer-peer social interactions are termed "social play" but also commonly referred to as rough-and-tumble play, and are thought to help build and develop adult sexual and social behavior patterns (Pellis & Pellis, 2009; Poole & Fish, 1976; G. T. Taylor, 1980; Van Den Berg, Hol, et al., 1999). Importantly, the changes observed have significant and translational relevance to neuropsychiatric disorders; which can include altered responses to drugs of abuse, hyperactivity in a novel environment, impaired sensorimotor gating, cognitive inflexibility, and social withdrawal and impaired social communication (Fone & Porkess, 2008; Hall, 1998; Lapiz, Fulford, Muchimapura, et al., 2003; Seffer, Rippberger, Schwarting, & Wöhr, 2015).

Social Play in Rats

Rough-and-tumble play is an activity that is engaged in voluntarily, is positively reinforcing, and is known to be energetic and vigorous in nature (Pellis & Pellis, 2009). Rough-and-tumble play contains rudiments of exaggerated or modified aggressive, sexual or predatory behaviors (Panksepp, Siviy, & Normansell, 1984; Pellis & Pellis, 2009; Vanderschuren, Achterberg, & Trezza, 2016; Vanderschuren, Niesink, & Van Ree, 1997). Therefore, the function is likely to prepare the participants for situations they will encounter as adults in which these behaviors will be exerted in a much more refined and polished manner, such as in dominance interactions and contests, as well as mating. Rough-and-tumble play is by no means the only form of play observed in the animal kingdom, for example, play can also involve objects such as a dog playing with a ball, or self-directed play such as a lamb gamboling through a meadow or a locomotor rotational play in mice (Pellis & Pellis, 2009). However, these other forms of play are lacking any social component crucial for development of the brain and appropriate social behavior and there is strong evidence supporting the necessity of play with social peers during development (Gruendel & Arnold, 1969; Hård & Larsson, 1968; Hol, Van Den Berg, Van Ree, & Spruijt, 1999; Lore & Flannelly, 1977; Pellis & Pellis, 2009; Poole & Fish, 1976; Seffer et al., 2015; G. T. Taylor, 1980; Van Den Berg, van Ree, & Spruijt, 1999).

In rats, rough-and-tumble play begins to emerge approximately three weeks after birth and gradually increases to its maximum peak frequency around four to six weeks, at which point playful interactions occur around 60 minutes per day, then decreases steadily around seven weeks to only a few minutes per day (Pellis & Pellis, 1991). During a period of rough-and-tumble the initiation of a playful attack, typically, is observed with one rat, the attacker, approaching the other and using its snout, attempts to nuzzle the nape of the other (Panksepp & Beatty, 1980; Pellis & Pellis, 2009; Poole & Fish, 1976; Vanderschuren et al., 2016, 1997). The nuzzling of the nape is known as “pouncing” or “nape contact” and is a key feature for the distinction between play and agonistic attacks (Pellis, 1988; Vanderschuren et al., 2016). If nape contact is successful then play-fighting will generally ensue, in which, constant attack and defense of the nape is the main goal. Several types of defensive maneuvers are utilized by rats in order to prevent their partner from gaining access to their nape. These tactics can differ depending on age and sex (Pellis & Pellis, 2009). Analysis of specific components involved in bouts of rough-and-tumble play vary depending on the specific research questions (Pellis &

Pellis, 2009), however, typically core components including pinning, wrestling and chasing are assessed. Pinning can be defined as one rat lying with its dorsal surface on the floor with the other rat standing over it. Wrestling, is rapid attack and defense of the nape and can encompass components of boxing, i.e., when two rats stand on their hind legs and rapidly push, paw and grab each other; and pouncing i.e., the solicitation of another rat by rubbing the nape of its neck. Additionally, chasing is also commonly assessed and is defined as one rat moving in the direction of, or pursuing the partner, while the partner is moving (Vanderschuren et al., 2016; Vanderschuren, Stein, Wiegant, & Van Ree, 1995). Specific behavioral components of rough-and-tumble play can be differentially regulated and can be selectively affected by pharmacological (Vanderschuren et al., 1995), brain (Heather C. Bell, Pellis, & Kolb, 2010; Schneider & Koch, 2005), prenatal (Raza, Himmler, Himmler, et al., 2015; Wellmann, George, Brnouti, & Mooney, 2015; Zaccaroni, Massolo, Della Seta, et al., 2017), genetic (Homberg, Schiepers, Schoffelmeer, Cuppen, & Vanderschuren, 2007) and environmental (Stockman & McCarthy, 2017; Vanderschuren et al., 1995) manipulations.

In a most basic and rudimentary way the brain structures involved in rough-and-tumble play can be divided into; the brain stem: which includes areas and neurotransmitter systems involved in regulating motivation and reward, i.e., the nucleus accumbens (NAcc) and hypothalamus; the subcortical structures, which includes the emotional centres, such as the amygdala; and the cortex which involves the higher cognitive functions of attention, planning, and working memory in areas such as, the frontal cortex and ACC (for comprehensive review see: (Pellis & Pellis, 2009; Vanderschuren et al., 2016)).

The brainstem itself is separated into three primary regions, the medulla, the pons, and the midbrain. Most prominently, the midbrain consists of several important structures involved in rough-and-tumble play (Pellis& Pellis. 2009; Vanderschuren et al, 2014). Within the upper midbrain exists the hypothalamus, in which neural circuits to and from are involved in regulating motivation and reward i.e. frequencies of playful attacks (Pellis & Pellis, 2009). Changes or damage to the hypothalamus or its circuits can lead to less interest and activity when engaged in play (Pellis & Pellis, 2009). The hypothalamus is also part of an intricate and complex set of direct and feedback influences via the HPA axis which is highly involved in the neurobiology of mood disorders such as MDD and BPD. The HPA axis controls reactions to stress and, some, early-life stress in mild or moderate forms appears to enable the development

of coping responses that allow adaptability and resiliency by enhancing HPA regulation (Gunnar et al., 2009; Lyons, Parker, & Schatzberg, 2010; Macrì, Granstrem, Shumilina, et al., 2009). However, when exposed to severe stress or trauma during early-life the HPA axis becomes hyper-reactive leading to increased and chronic release of stress hormones which can then contribute to the development of mood disorders and, thus, impair social behaviors (Flinn, Nepomnaschy, Muehlenbein, & Ponzi, 2011). Studies have shown that when rat pups are exposed to high levels of prenatal stress the expression of rough-and-tumble play is reduced and impaired (Ward & Stehm, 1991) and HPA reactivity is highly increased (Henry, Kabbaj, Simon, Le Moal, & Maccari, 1994). Interestingly, when provided with post-weaning enrichment during the critical period, effects of prenatal stress on play behavior can be reversed and HPA reactivity reduced, whereas, in rats prenatally stressed but given no enrichment there is no reduction in HPA reactivity (Morley-Fletcher, Rea, Maccari, & Laviola, 2003).

Another structure residing in the midbrain is the habenula which regulates monoaminergic neurotransmission (Lecourtier & Kelly, 2007) and is involved in reward and cognitive processes (Lecourtier & Kelly, 2007; Proulx, Hikosaka, & Malinow, 2014). In a study done by Vankerkhof and colleagues (van Kerkhof, Damsteegt, Trezza, Voorn, & Vanderschuren, 2013) juvenile rats are socially isolated for 24 hours show an increase in c-fos expression within the habenula, however, when given subsequent rough-and-tumble play the expression of c-fos decreases. This suggests that the habenula may mediate negative effects of social isolation and that this can be lessened with subsequent rough-and-tumble play.

The striatum has been shown to be correlated in rough-and-tumble play with species specific defense actions (Pellis & Pellis, 2009). When neonatal rats were given 6-OHDA injections into each lateral ventricle, the results were a severe disruption in organisation of rough-and-tumble play (Pellis, Castañeda, McKenna, Tran-Nguyen, & Whishaw, 1993). 6-OHDA, is characteristically used to lesion dopamine neurons. Additionally, pharmacological inactivation showed rough-and-tumble play behavior could be enhanced or increased by inactivation of NAcc core and dorsomedial striatum, respectively (van Kerkhof, Trezza, Mulder, et al., 2013). Several studies have confirmed the important role of dopamine action in rough-and-tumble play, showing that it has a role in both the motivation (Achterberg, van Kerkhof, Servadio, et al., 2016) and the pleasurable or stimulating effects of play (Manduca,

Servadio, Damsteegt, et al., 2016; Trezza, Baarendse, & Vanderschuren, 2009; Trezza & Vanderschuren, 2008).

Although, numerous subcortical structures may influence rough-and-tumble play as a result of projections and feedback from other areas (Vanderschuren & Trezza, 2013), one main focus has been in the role of the amygdala in the regulation of social behavior and rough-and-tumble play. The amygdala is commonly known as the integrative centre for emotions, emotional behavior and motivation (Cardinal, Parkinson, Hall, & Everitt, 2002) and evidence has been observed by means of a disruption in rough-and-tumble play when the amygdala is damaged (Daenen, Wolterink, Gerrits, & Van Ree, 2002; Meaney, Dodge, & Beatty, 1981). Moreover, it was shown that rough-and-tumble play is reduced in male rats but not in females after receiving amygdalar lesions (Meaney et al., 1981). This suggests along with more recent studies (Jessen, Kolodkin, Bychowski, Auger, & Auger, 2010; Kurian, Bychowski, Forbes-Lorman, Auger, & Auger, 2008; P. V. Taylor, Veenema, Paul, et al., 2012) that during developmental trajectories the development of the amygdala, in rats, can have an influence on the sex differences in rough-and-tumble play. The cortex is not an essential component in order for rough-and-tumble play to take place and neonatal rats with their cortex removed still develop rough-and-tumble play and do so at frequencies comparable to control rats (Pellis, Pellis, & Whishaw, 1992). However, where it does appear to be necessary to have a cortex is when it comes to specific defence tactics, i.e., rotating to a fully supine position versus only partially rotating but keeping one foot on the ground (Pellis et al., 1992). Similarly, more detailed cellular activity following rough-and-tumble play shows an increase in c-fos expression in the anterior cingulate cortex, prelimbic cortex, medial orbital and ventrolateral orbital cortex as well as a decrease of expression in the dorsolateral orbital cortex (van Kerkhof, Trezza, et al., 2013) suggesting that the role of the cortex in specific aspects of social play is much more intricate and complex than originally proposed.

Social Communication in Rats

Many rodents are known to emit ultrasonic vocalizations (USV) in numerous situations both social and non-social (Brudzynski, 2013, 2015; Portfors, 2007; Wöhr & Schwarting, 2013). They are classified as ultrasonic because they fall below the typical range of human hearing. Rats, are a common rodent model used to explore USV. However, unlike mice, rat USV have a more complex contextual use within social situations and alterations therein were suggested to be associated with social communication deficits seen in rat models of neuropsychiatric disorders. Shortly after birth rat pups begin emitting USV, as they develop into juveniles there becomes a separate distinction between USV made during negative, fearful or aggressive situations and those made during positive, social and rewarding situations.

Isolation-induced pup USV (40-kHz USV)

From birth to the time of weaning, rat pups emit USV in the range of 40-70 kHz (Hofer, Shair, & Brunelli, 2002; Noirot, 1968; Sales, 1972), commonly known as "40-kHz USV". The context of mother and pup separation has been thoroughly investigated subsequently is has been established that 40-kHz USV are emitted when pups are isolated from their mother or littermates (Brunelli & Hofer, 2001; Hofer, 1996; Insel & Winslow, 1991; Smotherman, Bell, Starzec, Elias, & Zachman, 1974; Wöhr & Schwarting, 2008a) and not as a result of a by-product of movement (Blumberg & Sokoloff, 2001). Using an established 40-kHz USV playback method can elicit retrieval behavior in mother rats (Wöhr & Schwarting, 2008a). Additionally, the number of USV emitted by pups in isolation has been negatively correlated with the maternal care given by the mother and this in turn affects processing of stress in adulthood (Schwarting & Wöhr, 2012) and has been linked to a high reactivity of HPA axis (Hennessy & Weinberg, 1990; Hofer, 1996; Tamborski Harvey & Hennessy, 1995). Thus, 40-kHz USV in infantile rats give the impression that they are emitted as an indicator for the pups' emotional state and of their attachment with the mother.

Fear-induced USV (22-kHz USV)

After weaning, there begins to be a distinction between two categories of USV emitted during differing social and non-social situations. The first is what is commonly referred to as "22-kHz alarm USV". Characteristics of 22-kHz USV include long call durations of 1000 ms

or more, a narrow frequency range typically between 18-24 kHz with low levels of frequency modulation, and typically, are seen to occur in bouts of two to eight calls (Wöhr et al., 2005). Various states of distress or negative affect are associated with 22-kHz USV, which can include inter-male aggression (Lore et al. 1976), stress responses (Borta, Wöhr, & Schwarting, 2006; Graham, Yoon, Lee, & Kim, 2009; Knutson, Burgdorf, & Panksepp, 2002; Van Der Poel & Miczek, 1991), and exposure to- or danger from predators (Blanchard, Blanchard, Agullana, & Weiss, 1991). As a result of 22-kHz USV appearing when in the presence of danger or in response to stress it was hypothesized that the USV are a reflection of the negative affective state of the rat (Brudzynski, 2013, 2015; Wöhr & Schwarting, 2013). The neural pathway connected to 22-kHz USV supports the idea that the USVs express and induce a negative affective state as it comprises the mesolimbic cholinergic system including the periaqueductal grey (PAG) and amygdala, which both are strongly involved in emotional regulation and house important components involved in defensive behaviors and fear (Brudzynski, 2013, 2015). Early on it was determined that 22-kHz USV were emitted when adult male rats were involved in inter-male aggression, and that the use of 22-kHz USV was as an appeasement signal by the subordinate rat when being attacked by a dominant (Lore, Flannelly, & Farina, 1976). Later it was further established that 22-kHz USV could be evoked as warning signals to other conspecifics when rats were exposed to predatory odors in an experimentally controlled, underground, colony system (Blanchard et al., 1991). Interestingly, 22-kHz USV emission was found to be potentiated by the presence of other conspecifics, implying that there is an audience effect. With further investigation it was suggested that the receiver of 22-kHz USV does not need to be in the presence of the predator to elicit defensive responses, simply hearing the alarm call may be enough to provoke a response (Blanchard et al., 1991), however, several subsequent studies have given conflicting results (Wöhr & Schwarting, 2008b). The social nature of 22-kHz USV, therefore, may be to convey warnings about any danger that the rats could be or are presently in, but the manner of how exactly is still under debate. Emerging from these studies is a growing interest in how rats communicate aversive situations to one another and what experiences are necessary in order to gain basic information and exhibit appropriate behavioral responses when 22-kHz USV are emitted. Conclusions so far have led researches to hypothesize that there is an auto-conditioning effect. This hypothesis states that context experience is required in order for 22-kHz USV to have an effect on the social

transmission of negative affective states. It was shown in studies using playback that when a sequence of previously recorded 22-kHz USV are presented, the result is a strong inhibition in locomotor activity (Burman, Ilyat, Jones, & Mendl, 2007; Endres, Widmann, & Fendt, 2007; Wöhr & Schwarting, 2007). However, this response in behavioral inhibition, or freezing, only appears if the receiver has been previously exposed to an aversive situation (Kim, Kim, Covey, & Kim, 2010). Parsana, Moran & Brown (2012) further confirmed the auto-conditioning hypothesis when playback of 22-kHz USV only resulted in freezing and behavioral inhibition in rats that had been previously exposed to aversive stimuli. Taken together one can infer that 22-kHz USV are learned via auto-conditioning and that prior exposure to aversive contexts is required in order to communicate affective states to conspecifics as well as, respond appropriately by generalizing future reactions in response to 22-kHz USV. Corroborating neurobiological mechanisms have been found to support the above hypothesis, as well as, indicate the role of 22-kHz USV as a measure of negative affective state (Brudzynski, 2013, 2015). Using c-fos to explore neural activity, Sadananda et al (2008) showed that after the playback of 22-kHz USV an increase in cellular expression was seen in basolateral amygdala and PAG. Moreover, increased activity in the amygdala was further observed during single cell recordings, following 22-kHz playback (Parsana, Li, & Brown, 2012). The rate of freezing is what is commonly measured during conditioned fear learning and, it turns out, the freezing rate is highly correlated with the emission of 22-kHz USV (Wöhr & Schwarting, 2008b). This response can be blocked, however, by inactivation of the amygdala (Brudzynski, 2013, 2015).

Pro-social interaction-induced USV (50-kHz USV)

The second distinctive USV emitted by rats, post-weaning, is 50-kHz USV, commonly referred to as “prosocial 50-kHz USV”. Typically, 50-kHz USV are emitted in short bursts less than 50 ms and can range in frequency from 31-90-kHz, although most occur between 50-70-kHz (Wöhr, 2018). There can be high rates of frequency modulation within single 50-kHz calls, with some lacking any modulation in frequency at all i.e. “FLAT calls”. Whereas, others exhibit high levels of modulation i.e., “TRILL calls” (Brudzynski, 2013; Burgdorf, Kroes, Moskal, et al., 2008; Wright, Gourdon, & Clarke, 2010). In an effort to understand rat vocal communication in a more in-depth manner, researchers have classified different subtypes of commonly observed 50-kHz USV (Burgdorf et al., 2008; Pereira, Andreatini, Schwarting, &

Brenes, 2014; Wright et al., 2010). A simplistic and early method of classification was simply to distinguish 50-kHz USVs into constant frequency (FLAT) or frequency modulated (FM) subtypes (Burgdorf et al., 2008). Using this system of classification FLAT calls included only a FLAT component whereas, FM calls also contain the FLAT 50-kHz component but included also either a TRILL and/or STEP component, and in 90% of FM calls typically two or more components are common (Burgdorf et al., 2008). Following the broad generalization of 50-kHz USV subtypes into either FLAT or FM, Wright et al. (2010) developed an extensive list of 14 different subtypes for 50-kHz USV. Using this method 50-kHz USV are classified based on structural components and detailed acoustic parameters. The list includes several variations of TRILL, FLAT and STEP calls, i.e., TRILL WITH JUMPS, FLAT-TRILL COMBINATION and STEP-DOWN/UP (Wright et al., 2010), to name a few. The idea behind such a large classification system would suggest that rats are potentially using specific call subtypes in specific situations. Recently specific subtypes from Wright et al (2010) have been linked to several behaviors. For example, in detailed investigations into adult male interactions Burke, Kisko, Pellis & Euston (2017) found that, specifically, FLAT calls are important to de-escalating aggressive interactions. Furthermore, FLAT calls serve as warning signals to keep away or risk being bitten (Burke, Kisko, Pellis, et al., 2017), which is in contrast to a purely submission signal hypothesized by other researchers (Lore et al., 1976; Sales, 1972; Sewell, 1967). Another classification system, established by Pereira et al (2014), has recently found a compromise in between the two ends of the spectrum. This classification system employs a rudimentary method but is still not as simplistic as Burgdorf et al (2008) nor as extreme as Wright et al (2010). The Pereira et al (2014) method classifies calls in the range of 50-kHz into four categories, including; FLAT, STEP, TRILL and MIXED. This method takes into account that there may be specific acoustic parameters that differentiate FM calls and, therefore, creates a distinction based on the rate of modulation within a certain call. For example, a STEP call is a fundamental FLAT call with at least one short FLAT element overlapping at the start or end of the call, importantly the STEP component of the call needs to be 5-kHz higher than the fundamental call. A TRILL on the other hand is a single call element with either one or two peak frequency changes higher than 5-kHz and changing in opposed directions at least 5-kHz apart, in other words, the call is zigzag shaped (Pereira et al., 2014). Additionally, Pereira et al (2014) classify FLAT calls in parallel with Burgdorf et al (2008) in that they are flat in structure

and maintain a constant frequency, and all remaining FM calls that do not fall within the STEP or TRILL categories are labelled as MIXED calls (Pereira et al., 2014).

Typically, 50-kHz USV occurs during various states of pleasure or positive affect (for comprehensive review see: (Brudzynski, 2013, 2015; Wöhr, 2018)). Positive social situations include: mating (Barfield & Thomas, 1986; Sewell, 2009), rough-and-tumble play (Himmler, Kisko, Euston, Kolb, & Pellis, 2014; Kisko, Euston, & Pellis, 2015; Kisko, Himmler, Himmler, Euston, & Pellis, 2015; Knutson, Burgdorf, & Panksepp, 1998; Lukas & Wöhr, 2015; Webber, Harmon, Beckwith, et al., 2012) and mimicking rough-and-tumble play through a human experimenter by means of tickling (Burgdorf & Panksepp, 2001; Mällo, Matrov, Herm, et al., 2007; Panksepp & Burgdorf, 2000, 2003; Schwarting, Jegan, & Wöhr, 2007) but also in some negative social interactions such as resident-intruder paradigms (Burgdorf et al., 2008). Also, non-social situations such as drug and food reward are known to elicit high levels of 50-kHz USVs (Burgdorf, Knutson, Panksepp, & Ikemoto, 2001; Pereira et al., 2014; Thompson, Leonard, & Brudzynski, 2006; Wöhr & Schwarting, 2008b). Two schools of thought currently exist as to the exact purpose of 50-kHz USV (Wöhr, Engelhardt, Seffer, Sungur, & Schwarting, 2015). The first theory is that 50-kHz USV are reflecting the positive emotional state of the sender, better known as the affective state hypothesis (Burgdorf et al., 2008; Knutson et al., 1998; Wöhr, Engelhardt, et al., 2015; Wöhr, van Gaalen, & Schwarting, 2015). The second theory is that they are used as social contact calls (Wöhr & Schwarting, 2007) and serve more of a practical communication purpose, meaning that the rats are using 50-kHz calls to communicate specific information to their conspecifics, for example, as play signals indicating that the sender is launching a playful attack directed at the partner (Kisko, Wöhr, Pellis, & Pellis, 2015). Importantly, neither the affective state, nor social contact call theories are mutually exclusive. It appears likely that 50-kHz USV serve both an affective and communicative function (Kisko, Wöhr, et al., 2015; Wöhr, Engelhardt, et al., 2015). Rat 50-kHz USV has been considered, by some, to be homologous with human laughter and has even been dubbed “Rat laughter” (Panksepp, 2005). It is thought that when rats emit 50-kHz USV they are expressing emotional states comparable to joy and happiness (Brudzynski, 2013; Knutson et al., 2002).

Evidence for Affective State

Importantly, the 50-kHz USV affective state hypothesis began to really emerge and take form twenty years ago when Knutson et al (1998) began investigating the role of appetitive 50-kHz USV throughout rough-and-tumble play. Through a series of experiments, the researchers determined that during play rats emit increased levels of 50-kHz USV and that they both correlated and predicted appetitive components of rough-and-tumble play. Primarily, 50-kHz USV were predictive of dorsal contacts in subsequent play sessions but were not related to previous play sessions, indicating that 50-kHz USV may be a more sensitive measure for pairwise play, and additionally suggesting that rats appear to retain and act on memories of prior rough-and-tumble interactions with the same partner (Knutson et al., 1998). Additionally, Knutson et al (1998) further went on to show that only one session of play was necessary to induce a motivational state which elicited 50-kHz USV emission, subsequently leading to the finding that 50-kHz USV were also increased in rats exposed to short term isolation and emitted in the anticipation of playful interactions (Knutson et al., 1998). Emission of 50-kHz USV in anticipation of rewarding playful interactions has since been repeatedly shown and can be taken as a strong indication of motivation for social play as well as other rewarding situations (Burgdorf, Knutson, & Panksepp, 2000; Burgdorf et al., 2008; Burke, Kisko, Swiftwolfe, Pellis, & Euston, 2017). In order to distinguish between merely just a state of general-arousal or by-products of increased locomotor behaviors (RW Bell & Nitschke, 1974), Knutson et al (1998) showed that, when tested under bright white light, conditioned 50-kHz USV calling is reduced, suggesting that it is appetitive-motivation driving 50-kHz emission. In a further effort to decouple the general-arousal hypothesis from the affective state hypothesis Burke et al (2017) demonstrated that during the anticipation of rough-and-tumble play a one-to-one relationship between 50-kHz USV emission and movements does not exist, casting further uncertainty as to general-arousal resulting in USV production. Since the initial study by Knutson et al (1998) numerous other studies investigating the role of 50-kHz USV in juvenile social play have been done strongly indicating that 50-kHz USV are highly correlated with social play behaviors (Burgdorf et al., 2008; Burgdorf, Panksepp, & Moskal, 2011; Himmler et al., 2014; Kisko, Euston, et al., 2015; Kisko, Himmler, et al., 2015; Lukas & Wöhr, 2015; Vanderschuren et al., 2016, 1997; Webber et al., 2012). Along with rough-and-tumble play another method eliciting high levels of 50-kHz USV and thought to be reflective of the

positive emotional state of the sender, is tickling. During a bout of tickling a human hand is used to mimic hetero-specific rough-and-tumble play (Mällo et al., 2007; Panksepp & Burgdorf, 2000, 2003; Schwarting et al., 2007). The rate of 50-kHz USV emission during a bout of tickling can be increased by short durations of isolation, as well, rats that emit high numbers of 50-kHz USV show shorter latencies when approaching a human hand and, also, express high levels of 50-kHz USV when given cues associated with tickling, i.e. the experimenters hand, demonstrating that there is a high association for social motivation and increased positive affective states indicated by the 50-kHz USV emission rates (Panksepp & Burgdorf, 2000, 2003).

Non-social means of eliciting 50-kHz USV is often done through the administration of psychostimulants such methamphetamine (Mahler, Moorman, Feltenstein, et al., 2013) and cocaine (Barker, Bercovicz, Servilio, et al., 2014; Barker, Root, Ma, et al., 2010; Barker, Simmons, Servilio, et al., 2014; Browning, Browning, Maxwell, et al., 2011; Ma, Maier, Ahrens, Schallert, & Duvauchelle, 2010; Maier, Abdalla, Ahrens, Schallert, & Duvauchelle, 2012; Maier, Ahrens, Ma, Schallert, & Duvauchelle, 2010; Meyer, Ma, & Robinson, 2012; Mu, Fuchs, Saal, et al., 2009; Williams & Undieh, 2010; Wright, Dobosiewicz, & Clarke, 2012), although more frequently, through amphetamine (Burgdorf et al., 2001; Engelhardt, Fuchs, Schwarting, & Wöhr, 2017; Knutson, Burgdorf, & Panksepp, 1999; Lehner, Taracha, Kaniuga, et al., 2014; Pereira et al., 2014; Taracha, Kaniuga, Wyszogrodzka, et al., 2016; Thompson et al., 2006; Wright et al., 2012); but for comprehensive review see: (Rippberger, van Gaalen, Schwarting, & Wohr, 2015)). Psychostimulants, specifically amphetamine, are known to cause elevated positive mood in humans and this is thought to be reflected in the dramatic increase in 50-kHz USV emission in rats, followed by an increase in hyper-locomotion (Rippberger et al., 2015). Moreover, amphetamine induced 50-kHz USV have been suggested to represent translational markers for mania-like positive affective states and can be reduced by treating with the standard “mood-stabilizer” lithium, in rats (Pereira et al., 2014; Wendler, de Souza, Vecchia, et al., 2016) which supports the idea that 50-kHz USV emission may be representative of the affective state of the rat. Studies using social play (Himmler et al., 2014; Kisko, Euston, et al., 2015; Kisko, Himmler, et al., 2015; Knutson et al., 1998; Lukas & Wöhr, 2015; Webber et al., 2012), tickling, (Burgdorf & Panksepp, 2001; Mällo et al., 2007; Panksepp & Burgdorf, 2000, 2003; Schwarting et al., 2007), and psychostimulant administration (Burgdorf et al.,

2001; Rippberger et al., 2015; Thompson et al., 2006) all support the notion that 50-kHz USV emission is to express the affective state of the rat possibly because the regulation and organization of 50-kHz USV emission within the rat brain is linked to reward systems.

Evidence for Social Contact Call

The idea that 50-kHz USV act as social contact calls has been around for quite some time, in the early days of USV research it was mainly studied in sexual contexts and was thought to be emitted in order to maintain appropriate sexual social contact (Barfield & Thomas, 1986). More recently, within same-sex studies it was found that rats spend more time with conspecifics emitting high levels of 50-kHz USV over those that emit low levels (Panksepp, Gordon, & Burgdorf, 2002) and when given an operant task they will work for a chance to be exposed to 50-kHz USV playback (Burgdorf et al., 2008). It has been repeatedly shown, using an eight-arm radial maze, that playback via an ultrasonic loudspeaker consistently promotes social approach behavior in response to 50-kHz USV, but not white noise (Seffer et al., 2015; Willadsen, Seffer, Schwarting, & Wöhr, 2014; Wöhr, Engelhardt, et al., 2015). Moreover, work by Brudzynski and Pniak (2002) demonstrated that 50-kHz USV emission is driven by the desire for social contact elicited by exposure to the odour of conspecifics, i.e. 50-kHz USV emission rates are highly correlated with the number of rats that leave their odor. Notably, Knutson et al (1998) observed that rates of 50-kHz USV emission and dorsal contacts decreased after the first minute of play, whereas pins and other playful behaviors took longer to decrease in frequency, clearly demonstrating a relationship between USV and dorsal contact behavior. A detailed study by Himmler et al (2014) provided a comprehensive temporal analysis of 50-kHz USV during rough-and-tumble play which indicated that rats are more likely to emit 50-kHz USV directly before launching a playful attack rather than immediately following the attack and that specific 50-kHz USV subtypes are associated with specific behaviors. This suggests that 50-kHz USV during rough-and-tumble play are possibly being used as play signals in order to maintain and promote playful contact (Himmler et al., 2014). More recently, Burke et al (2017) found specific 50-kHz USV subtypes were associated with specific behaviors, namely running and jumping, during the anticipation of rough-and-tumble play in juvenile rats. In an additional study done in adults Burke et al (2017) further found an association between the FLAT 50-kHz USV subtype and an escalation of aggressive behavior.

Conversely, however, several studies have failed to find evidence that 50-kHz calls are attractive to other rats in a communicative function. For example, playback of 50-kHz calls during mating interactions appears to have no effect on the attractiveness of the partner, either male or female (Snoeren & Ågmo, 2013; Thomas, Howard, & Barfield, 1982; Thomas, Talalas, & Barfield, 1981) and even supports a self-regulation hypothesis in that, the absence of female vocalizations appears to affect the females' own behavior and not the males' behavior (Nicholas R. White & Barfield, 1987).

Neurobiology of 50-kHz USV

Many neurotransmitter systems are involved in 50-kHz USV production, but particularly important and extensively studied is dopamine (Brudzynski, 2013, 2015). Midbrain dopamine has an essential role in the acquisition of natural reward as well as drug-seeking behavior (Spanagel & Weiss, 1999) and dopaminergic projections in the VTA innervate the NAcc, as well as the amygdala, hippocampus, mPFC and ventral pallidum which play important roles in regulating information through the limbic circuits (Pierce & Kumaresan, 2006). Importantly, the neuronal firings of VTA neurons have been associated with the synaptic release of dopamine in the NAcc (Sombers, Beyene, Carelli, & Wightman, 2009) which then contributes directly to the generation of a positive emotional state and subsequent production of 50-kHz USV (Brudzynski, 2013, 2015). This result is clearly evident when administering amphetamine in rats, after which, the catecholaminergic systems are affected through the direct interaction with dopamine and noradrenaline transporters which results in an increase in extracellular dopamine, and noradrenaline, concentration thought to then result in a high arousal and an anticipation of emotionally positive outcomes (Hutson, Tarazi, Madhoo, Slawecki, & Patkar, 2014). This is reflected in conditioned place-preference paradigms, in which rats emit more 50-kHz USV when in places where they have previously received amphetamine (Ahrens, Nobile, Page, et al., 2013; Knutson et al., 1999). This is additionally supported by showing that electrical stimulation of the mesolimbic pathways of the brain, as well as, the anticipation of oncoming stimulation, elicits 50-kHz USV (Burgdorf et al., 2000). Dopamine increase is also highly linked with rough-and-tumble play (for review see: (Vanderschuren et al., 2016, 1997)) which, as mention briefly above, is strongly coupled to 50-kHz USV emission (Burgdorf et al.,

2008; Himmler et al., 2014; Kisko, Euston, et al., 2015; Kisko, Himmler, et al., 2015; Kisko, Wöhr, et al., 2015; Knutson et al., 1998; Webber et al., 2012).

Dopamine release is also, evident not only in the sender (Burgdorf et al., 2000; Burgdorf, Wood, Kroes, Moskal, & Panksepp, 2007) but also in the recipient of 50-kHz USV (Willuhn, Tose, Wanat, et al., 2014). In a recent and novel experiment, freely moving rats were exposed to playback of 50-kHz USV, 22-kHz USV, time and amplitude matched white noise and background noise in a random order and using fast-scan cyclic voltammetry Willuhn et al (2014) recorded dopamine signalling in the NAcc. Only the presentation of 50-kHz USV, however, induced phasic dopamine release coupled with approach behavior towards the ultrasonic speaker (Willuhn et al., 2014). This is important for several reasons, first it demonstrates that there is a clear neuroanatomical region for 50-kHz USV that is distinct from 22-kHz USV, evidenced in the lack of NAcc dopamine release during the playback of 22-kHz USV and also it emphasizes a functional link between pro-social communication and reward-related neurotransmission (Willuhn et al., 2014). Second this finding is important because it adds additional evidence for a more social contact call function for 50-kHz USV. Willuhn et al (2014) establishes this by showing simply that acoustic stimuli indicating the presence of a conspecific rat, i.e., 22-kHz USV and the sound of a rat moving on cage bedding, was not enough to elicit dopamine release. Rather, the phasic dopamine release in NAcc and the approach behaviour were only seen in response to 50-kHz USV playback suggesting that it's the actual communicative signal emitted by the conspecific rat that elicits the dopamine release.

In addition to the NAcc and VTA, the level of neurogenesis within the hippocampus has been linked to the regulation of affective states. Studies have shown that aversive stimuli reduce 50-kHz USV (Panksepp & Burgdorf, 2003) and also hippocampal cell proliferation (Czéh & Lucassen, 2007). Notably, proliferation is necessary for antidepressant effects to take place during selective serotonin reuptake inhibitor (SSRI) treatment (Santarelli, 2003). Bearing this in mind, Wöhr & Schwarting (2009) investigated the effects on hippocampal cell proliferation after exposing rats to tickling. An increase in cell proliferation indicates an increase in affective state, and 50-kHz USV emission during tickling was highly correlated with increases in hippocampal cell proliferation (Wöhr & Schwarting, 2009). Moreover, the increase in proliferation was strongly elevated in rats that had high 50-kHz USV emission

during tickling whereas, in contrast, rats that had low levels of 50-kHz USV emission had proliferation levels comparable with the control, non-tickled, rats (Wöhr & Schwarting, 2009). Suggesting that rats emitting high levels of 50-kHz USV during tickling, more than likely, experienced the situation as highly pleasurable and rewarding. The difference in high and low levels 50-kHz USVs during tickling supports other findings demonstrating that there can be interindividual variation within rats on the rate of USV emissions (Mällo et al., 2007; Schwarting et al., 2007). By selectively breeding separate lines for high and low calling rats Burgdorf et al (2005) found that the low-calling rats had deficits in early social motivation and rough-and-tumble play (Harmon, Cromwell, Burgdorf, et al., 2008; Webber et al., 2012) similar to animal models of autism (Moskal, Burgdorf, Kroes, Brudzynski, & Panksepp, 2011). This suggests that there may be a difference in positive, as well as negative, emotional phenotypes distinguishing specific genes involved in regulating emotional learning and this could lead to impairments or alterations during critical periods of development.

Evidence from Devocalization and 50-kHz Playback Studies

The affective state theory can be further supported by studies using devocalization in juvenile rats, done by cutting a small section of the laryngeal nerves. When the ability to emit 50-kHz USV is removed, rough-and-tumble play is subsequently altered in several ways, most notably, in pairs where both animals are devocalized the frequency of playful interactions are severely reduced. However, the decrease in playful behaviors can be returned to level comparable to controls, by pairing a devocalized rat with a vocal partner (Kisko, Himmler, et al., 2015). The ability to emit 50-kHz USV, therefore, appears to be related to the positive affective state of the sender, i.e., the one emitting USVs, and thus, abolishing USV production may interfere with the motivation for playful interactions by possibly changing the rewarding value gained from playing. However, it also appears likely that simply hearing 50-kHz USV is enough to stimulate reward and promote playful interactions. In recent studies, it has been shown that playback of 50-kHz calls activates parts of the NAcc, as well as the frontal and motor cortices (Sadananda et al., 2008). These areas are associated with emotion, meaning that the 50-kHz USV could be capable of inducing or changing the emotional state of the receiver, as is evident in pairs of rats in which one is vocal and the other devocalized (Kisko et al, 2015a). Additionally, it was found that when intact, meaning vocal, rats were housed with devocalized

cage mates during the peak play period, the intact rats showed a severe decrease in frequency of playful interactions and subsequent 50-kHz USV emission (Kisko, Wöhr, et al., 2015) suggesting that this period of development is not only important to proper development of social behavior but also to associated 50-kHz USV. Therefore, when intact rats play with devocalized cage mates, they may not get the necessary feedback from 50-kHz USV needed during this period essential to learning the affiliative value of 50-kHz USV during social interactions.

Evidence from 50-kHz USV playback supports a critical learning period for USV during the peak play phase, in addition to, providing further indication that 50-kHz USV may also have a more communicative function. A study done by Seffer et al (2015) established that the length of social isolation and re-socialization during the critical period can have a significant impact on response to USV playback. By using the exposure to one of three experimental housing conditions for four weeks: no isolation, group housing; short term isolation, 24 hours before testing; and long-term isolation, where rats were isolated 28 days before testing, the response to pro-social 50-kHz USV playback was investigated. Interestingly, group exposed to long-term isolation showed no approach behavior in response to pro-social 50-kHz USV and in fact the response seen in the group was that of behavioral inhibition. Further investigate into the observed phenomenon by exposing long-term isolated rats to one additional week of peer rearing, established that the behavior in response to 50-kHz USV could be rescued. It, therefore, seems apparent that during the critical period, rats not only need to engage in rough-and-tumble play and other social interactions in order to appropriately navigate social situations but that the concomitant USV emission is also an essential component in the emotional learning and development and that experience with contextual use of USV enable the rats to respond appropriately to emotionally valanced stimuli.

The need for proper contextual and emotional learning when responding to emotional stimuli is evident in further devocalization studies (Kisko et al, 2015b), in which the inability to produce USV often resulted in aggressive social interactions more than when USV production was intact. The tendency for the situation to escalate into an aggressive encounter only when one rat was devocalized indicates that the use of USV are necessary to create a less intense emotional state, furthermore it may provide insight into a communication value for USV. Often used to reduce aggression, appeasement signals are thought to maintain friendly

relationships among group members (Bradbury & Vehrencamp, 2011). It is well known that in a novel arena two unfamiliar rats will compete for the dominant role, generally the competition involves a form of rough-and-tumble play that gradually will become rougher as a way to establish dominance (Pellis & Pellis, 1987). Once dominance has been established, the submissive rat will produce 22-kHz calls (Assini, Sirotin, & Laplagne, 2013; Portavella, Depaulis, & Vergnes, 1993; Sales, 1972), possibly functioning to inhibit any further attacks (Lore et al., 1976; Sales, 1972). Likewise, 50-kHz calls are produced by the intruder in resident-intruder paradigms (Burgdorf et al., 2008), suggesting that when confronting an unfamiliar animal, it is the 50-kHz calls that are being used to appease the resident and inhibit any further attacks (Takahashi, Thomas, & Barfield, 1983). In this way, the sender is utilizing USV to communicate with the receiver in order to prevent further attacks and supporting a more communicative role in certain situations.

As a tool for uncovering phenotypes or mechanisms of neuropsychiatric disorders, particularly affect impairments and social communication deficits, USV are important for translational rodent models (Burgdorf et al., 2008), specifically rats, in which a highly social lifestyle is essential to proper development and social functioning in adulthood (Pellis & Pellis, 2009). So far, it has been shown that 50-kHz USV, likely, reflect the positive affective state of the rat and thus serve a socio-affective function (Kisko, Wöhr, et al., 2015; Wöhr, 2018; Wöhr, Engelhardt, et al., 2015).

Cognition, Learning and Memory

Studies looking into neuropsychiatric disorders such as SCZ, MDD and also ASD, tend to focus on the overt i.e. positive and affective symptoms, however, cognitive dysfunction has recently been noted to be equally as important and plays a vital role in long-term outcomes and the overall quality of life for patients (Kurtz, 2005; Young, Powell, Risbrough, Marston, & Geyer, 2009). In regards to MDD, the DSM-5 (American Psychiatric Association, 2013) describes a severe decline in the capacity to function for the individual. Cognitive symptoms can be wide ranging and encompass many different processes such as perception, attention, working and long-term memory, executive function, social cognition and language. Due to increased knowledge of the neurocircuitry and advancements in methods, animal models, specifically rodents and non-human primates, have been beneficial to dissecting the underlying cognitive mechanisms and endophenotypes implicated and altered in patients suffering from neuropsychiatric disorders (for comprehensive review see: (Keeler & Robbins, 2011)).

In particular relevance to SCZ and MDD, one highly employed behavioral assay is spatial and reversal learning (Olton & Paras, 1979; Olton & Samuelson, 1976). In spatial learning, one of two assays are generally used. The first is the MWM (Morris, 1981), where rats or mice learn the location of a hidden platform, and second is a spatial radial maze in which, the task is for rats to remember the constant location of food baited arms in an 8-arm radial maze (Olton & Paras, 1979). The MWM assesses mainly the animal's spatial memory capabilities, whereas the radial maze assesses spatial, as well as, working and short-term memory capabilities. Both assays are similar to the CANTAB paired associates learning task in humans, in which the goal is to remember and locate several abstract visual objects over short delays (Sahakian, Morris, Evenden, et al., 1988; Swainson, Hodges, Galton, et al., 2001). This task is sensitive to mild cognitive impairments observed in SCZ (J. H. Barnett, Sahakian, Werners, et al., 2005). The hippocampus is well known to be engaged in tasks such as this and has been observed in GWAS studies strongly implicating *CACNA1C* in neuropsychiatric disorders there are alterations in hippocampal activation and functioning (Dietsche et al., 2014; Erk, Meyer-Lindenberg, Schmierer, et al., 2014; Krug et al., 2014; Paulus et al., 2014). Reversal learning can further be used to measure several negative aspects of neuropsychiatric disorders, such as compulsivity and emotional regulation. Compulsive behavior can often be coupled with impulsive behavior, which is behavior that is premature and results as a consequence of the inability to wait, in

contrast compulsive behavior perseveres abnormally (Keeler & Robbins, 2011). In reversal learning paradigms this is measured by persistent responding to the formerly reinforced stimulus when the previously reinforced stimulus becomes correct or, during extinction periods when the reward is eliminated entirely. Moreover, this form of working memory and spatial learning for stimulus locations likely involves the PFC (Kolb, 1984) which is implicated in emotional regulation because of its abundant connections to areas such as ACC and amygdala (Ray & Zald, 2012). There are indications that depressed individuals have exaggerated and often catastrophic reactions to negative feedback, which subsequently has a strong impact on cognitive functioning (Elliott, Sahakian, McKay, et al., 1996). In a task of reversal learning in which the correct choice is rewarded 80% of the time and negative feedback provided the remaining 20%, patients with depression make inappropriate shifts in response choice following false negative feedback (Taylor Tavares, Clark, Furey, et al., 2008). This has been replicated in rats by manipulating the levels of serotonergic functioning, showing that when this function is reduced the effects mimic that seen shift-responses in individuals with depression (Bari, Theobald, Caprioli, et al., 2010). Another form of memory impairment often seen in MDD and SCZ is recognition memory (Dere, Pause, & Pietrowsky, 2010). Consisting of two main components, recognition memory includes a recollective (episodic) and familiarity component (Squire, Stark, & Clark, 2004). A well-known assay to measure this in animals is through the novel object recognition test, in which the animal explores an object during a sample trial and is then given a choice between a novel and familiar object (Ennaceur & Delacour, 1988). The time spent exploring the new versus old object is a measure of familiarity, or recognition, as well as curiosity and is thought to be homologous with the MATRICS visual learning and memory domain used in humans (Young et al., 2009). Several studies have indicated that deficits in recognition memory are evident in rodent models of SCZ and MDD (Grayson, Leger, Piercy, et al., 2015; Markham, Taylor, Taylor, Bell, & Koenig, 2010; Wilson & Terry, 2013).

Albeit, not an exhaustive list, the descriptions above outlining some forms of behavioral assays used to test cognition, learning and memory with regards to animal models, specifically rodents, allows us to gain insight into the numerous methods that are available when trying to parse the connections between impairments seen in neuropsychiatric disorders and the underlying mechanisms and neurocircuitry involved. It has been suggested that the current

treatment plans for affective and positive symptoms may, in fact, exacerbate the cognitive deficits in some patients (Wallace, Ballard, Pouzet, Riedel, & Wettstein, 2011), reinforcing a need for effective animal models for treatment encompassing all domains of impairment.

Objectives and Hypothesis

GWAS and clinical studies focusing on the *CACNA1C* risk gene in humans, as well as *Cacna1c* mouse models, suggest that this gene is highly implicated in neuropsychiatric disorders such as MDD, BPD, SCZ and also ASD, all of which have core features that include impairments in social behavior and social communication as well as cognitive deficits. In this dissertation, the main objective, using the newly developed heterozygous *Cacna1c* rat model, is to investigate the role *Cacna1c* plays, particularly, in the development of social behavior and concomitant ultrasonic communication, but also in areas of cognitive deficits.

To this aim, in Review I, the devocalization of juvenile rats provides compelling evidence for a cooperative association between 50-kHz USV and social play behaviors. Robust emphasis on the relationship between social play behavior and concomitant 50-kHz USV is important because, a key parameter to assess ultrasonic communication in the *Cacna1c* rat model (Studies I and II) is through juvenile rough-and-tumble play. Several studies investigating USV in rats during social interactions suggests that the functional role of USV may be to communicate specific information during specific contexts for example, during rough-and-tumble play (Himmler et al., 2014; Knutson et al., 1998). In contrast however, other studies have shown no evidence for a direct one-to-one relationship between USV and social behaviors, indicating that the functional role is more to express the affective state of the sender (Kisko, Euston, et al., 2015; Kisko, Himmler, et al., 2015; Snoeren & Ågmo, 2013). Therefore, in this thesis, the purpose of Review I, is to take an in-depth look at the role 50-kHz USV play for juvenile and adult rats while engaged in social interactions, specifically rough-and-tumble play. It is expected, that Review I important information is provided about the role of 50-kHz USV during juvenile rough-and-tumble play by means of devocalization techniques. During juvenile social interactions the relationship to 50-kHz USV is of particular relevance because rough-and-tumble play and concomitant 50-kHz USV emission was a core assessment in our *Cacna1c* rats in Study I and Study II.

The role of USV as social signals, the emotional background and the underlying brain mechanisms implicated have been studied for several years and there is now a substantial yet ever growing body of literature (Brudzynski, 2013, 2015). In Review I, as well as Study I and II, the role of USV in juvenile and adult rats was the prime focus, however, Review II is

principally focused on the investigation of USV by means of playback techniques which is another central assessment applied to our juvenile *Cacna1c* rats in Studies I and II. The playback method developed by Wöhr & Schwarting (2007) has received considerable attention and provides a major approach to analyze socio-communicative functions of USV. It is expected that an in-depth review of USV in relation to playback techniques provides substantial information about the neural correlates involved in USV production and reception as well as expected behavioral responses to USV playback of relevance for our *Cacna1c* rat model, which is expected to display social deficits.

In recent studies, conflicting evidence has been given as to the exact role for *Cacna1c* in social impairments seen in mouse models (Bader et al., 2011; Dedic et al., 2017; Kabir et al., 2017). However, sociability in mice is not as highly valued as it is in rats and therefore, this particular animal model may not be the best choice to investigate the potential role for *Cacna1c* in the development of social behavior and communication. For this purpose, in Study I, social behavior, with particular emphasis on rough-and tumble play in juvenile male *Cacna1c* heterozygous rats, in comparison to wildtype littermate controls, was assessed during the critical period in which playful interactions are most important to development. At the affective and pro-social communication level, emission of 50-kHz USV was measured and analyzed in detail to find out if there were alterations in specific components of pro-social communication that may contribute to impairments in social behavior of *Cacna1c* heterozygous juvenile male rats. It is expected that in rough-and-tumble play behavior, deficits in the frequency of playful interactions are expected in a genotype-dependent manner further reflected by altered 50-kHz USV emission at the communication level. Characteristics and subtypes of 50-kHz USV are further expected to differ between genotypes. In terms of response to playback of 50-kHz USV it is expected that *Cacna1c* haploinsufficiency will result in an impaired social approach response.

Sex-differences were previously reported in *Cacna1c* heterozygous mice (Dao et al., 2010; Zanos, Bhat, Terrillion, et al., 2015). However, it is not known whether these differences are also evident in social situations. Furthermore, social communication, in terms of USV has not yet been reported in social interactions of *Cacna1c* mouse models in either males or females. To answer these questions, Study II has focused on sex-differences resulting from *Cacna1c* haploinsufficiency on social behavior, specifically rough-and-tumble play and concomitant 50-

kHz USV emission, as well as response to 50-kHz USV playback. It is expected that rats that are *Cacna1c* haploinsufficient will show alterations in play behavior in a sex-dependent manner, further reflected in 50-kHz USV emission rates when compared to wildtype littermate controls. Additionally, sex-dependent effects are expected in both behavior and 50-kHz USV emission, based on previous studies showing differences in the frequencies of male and female rough-and-tumble play and 50-kHz USV emission (Himmler et al., 2014; Pellis & Pellis, 2009). Playback of 50-kHz USV is expected to, again, show impairments in *Cacna1c* heterozygous rats regardless of sex.

Cacna1c mouse models have recently began to corroborate evidence for hippocampal alterations found in *CACNA1C* risk allele carriers rs1006737 (Bader et al., 2011; Lee et al., 2016; Moosmang et al., 2005; Temme et al., 2016; J. A. White et al., 2008) suggesting that, as in humans, *Cacna1c* may play a role in cognition, learning and memory. Therefore, in Study III cognitive phenotypes were measured using tasks designed to specifically test working memory, episodic memory, and recall or recognition memory in haploinsufficient *Cacna1c* adult rats in comparison to wildtype littermate controls. It is expected that *Cacna1c* heterozygous rats will to show impairments in aspects of memory related to cognition during both a working memory task such as spatial learning and re-learning as well as during recall and recognition memory in both spatial memory tasks and object recognition tasks.

Publications

Summary of Publications

Review I: From Play to Aggression: High Frequency 50-kHz Ultrasonic Vocalizations as Play and Appeasement Signals in Rats.

Based on previous findings, evidence suggests that 50-kHz USV are maintaining and promoting playful interactions in juvenile rats, however, as adults 50-kHz USV serve more of a communicative, appeasement role (Kisko, Euston, et al., 2015; Kisko, Himmler, et al., 2015). Thus, 50-kHz USV likely, act as socio-affective communication to both indicate the affective

state of the sender as well as convey information to the receiver. This review, therefore, aims to highlight the importance of 50-kHz USV emission during rough-and-tumble play and further outlines impairments resulting from the absence of 50-kHz USV. Moreover, information about the crucial role of environment and peer-peer interactions during the critical stage after weaning are emphasized. New data on the role of 50-kHz USV during specific rough-and-tumble play behaviors, namely pinning reveals that it is the partner doing the pinning that is possibly gaining the most reward. This finding is in contrast to long-held ideas suggesting that the act of being pinned is most rewarding to rats. The essential relationship between 50-kHz USV and rough-and-tumble play, as well as the impairments resulting from the absence of either component is important because it is a key method to discover impairments in development of social behavior and communication, with relevance to neuropsychiatric disorders. For these reasons social play and concomitant 50-kHz USV emission was assessed in the juvenile *Cacna1c* rat model used in Study I and II.

Review II: Playback of Ultrasonic Vocalizations to Juvenile and Adult rats: Behavioral and Neuronal Effects

Providing a substantial update on the most recently published findings (Wöhr and Schwarting, 2010), this review takes a detailed look into the effects of ultrasonic playback on behavioral and neuronal responses in rats. By means of both artificial, as well as natural 22- or 50-kHz USV, the importance and emotional relationship of USV to social approach and avoidance behavior is established, with additional links to associated brain pathways and neurotransmitter release. Of relevance to preclinical models of depression, a cognitive bias is observed in response to playback suggesting that USV playback influences the rats' emotional states in terms of "emotional contagion" with 22-kHz leading to a negative emotional state and 50-kHz leading to a positive one. Furthermore, juvenile social deprivation; a typical method modeling neurodevelopmental disorders, impairs behavioral approach responses to 50-kHz USV playback (Seffer et al., 2015). Taken together, evidence from 22- and 50-kHz USV playback provides an important translational approach to investigate disease-relevant phenotypes, particularly those linked to *CACNA1C*, in which social deficits play a key role. For these reasons the 50-kHz USV playback method is emphasized to be an essential component in the assessment of juvenile social behavior in *Cacna1c* rats in Study I and II.

Study I: *Cacna1c* Haploinsufficiency Leads to Pro-Social 50-kHz Ultrasonic Communication Deficits in Rats

Several studies have linked mutations in the *CACNA1C* gene to ASD and ASD-like disorders, such as Timothy Syndrome (Bader et al., 2011). Additionally, MDD, BPD, and SCZ have also been linked to *CACNA1C* (Bhat et al., 2012). Importantly, key characteristics of these disorders include social behavior and communication impairments. For example, ASD is typically characterized by early childhood onset with atypical development and exists with a 4:1 ratio in males over females (Lai et al., 2014). The typical period of onset is also the stage when social play behavior is most important and influences the development of adequate social and communication abilities in adulthood. This prominent period of time, interestingly, is also important in the development of juvenile rats, which are known to be a highly gregarious species. Studies showing social deprivation during this critical period of development can result in disorder-like behavioral phenotypes and impairments in ultrasonic communication. Study I, investigates the association of social development impairments in disorders linked to *CACNA1C* with particular focus on the critical period of social behavior and communication in juvenile males. Using established methods outlined in Review I and II, rough-and-tumble play, 50-kHz USV emission and 50-kHz USV playback were assessed. Results show that in *Cacna1c*$^{+/-}$ and *Cacna1c*$^{+/+}$ males, social play behavior is unimpaired. However, prominent deficits in *Cacna1c*$^{+/-}$ males are apparent in both the production of 50-kHz USV and in the behavioral social approach response to 50-kHz USV playback. Most notably, 50-kHz USV production in *Cacna1c*$^{+/-}$ males is significantly lower than in wildtype controls and is characterized by higher peak frequencies, 70-90-kHz, and lower peak amplitude. Moreover, the 50-kHz USV subtype profile for *Cacna1c*$^{+/-}$ differed from *Cacna1c*$^{+/+}$ littermate controls with a strong reduction of FLAT and MIXED 50-kHz USV seen *Cacna1c*$^{+/-}$ males compared to the wildtype controls. Deficits in social communication may indicate that, in males having only one copy of the *Cacna1c* gene results in impairments in the emotional incentive salience for pro-social 50-kHz USV in both the sender and the receiver.

Study II: Sex-Dependent Effects of *Cacna1c* Haploinsufficiency on Juvenile Social Play Behavior and Pro-Social 50-kHz Ultrasonic Communication in Rats

Findings from *Cacna1c* mouse models have supported sex-specific mechanisms seen in humans carrying the *CACNA1C* rs1006737 risk allele (Dao et al., 2010; Strohmaier et al., 2013). Study I assessed only male juvenile *Cacna1c* rats, Study II, however, is interested in the sex-specific effects and differences in juvenile rough-and-tumble play, 50-kHz USV emission and 50-kHz USV playback. Results show that indeed there is a sex-specific effect in terms of rough-and-tumble play and 50-kHz USV emission. During rough-and-tumble play, the female *Cacna1c*$^{+/-}$ rats differ from both the *Cacna1c*$^{+/+}$ female littermate controls as well as, the *Cacna1c*$^{+/+}$ males. This difference is primarily driven by an increase in pinning behavior in *Cacna1c*$^{+/-}$ females. Interestingly *Cacna1c*$^{+/+}$ females have rates of rough-and-tumble play comparable to the *Cacna1c*$^{+/+}$ males. This is in disagreement to studies indicating sex differences in juvenile social play (Argue & McCarthy, 2015). In 50-kHz USV emission female *Cacna1c*$^{+/-}$ and *Cacna1c*$^{+/+}$ rats had decreased USV emission in comparison to the male *Cacna1c*$^{+/+}$ littermate controls. During 50-kHz USV playback, irrespective of genotype, females responded with social approach behavior and a preference for the arms closest to the speaker during 50-kHz USV presentation. Nonetheless, *Cacna1c*$^{+/-}$ females were similar to *Cacna1c*$^{+/-}$ males in that no preference for proximal or distal arms was evident in the minutes following 50-kHz USV playback. Sex-specific effects indicate that a having only one copy of the *Cacna1c* gene influences males and females in a differential manner. In addition, general motor and olfactory assessments were performed to rule out confounding factors. Irrespective of genotype, no differences were apparent in either male or females in terms of general motor function or olfactory capabilities. Similarly, repetitive and stereotyped patterns of behavior, revealed that irrespective of genotype no ASD-like phenotypes were apparent in males or females.

Study III: Sex-Specific Effects of *Cacna1c* Haploinsufficiency on Object Recognition Memory, Spatial and Reversal Learning Capabilities in Rats.

This study explores the effects of *Cacna1c* haploinsufficiency on cognitive functioning in adult male and female *Cacna1c* rats. Specifically, paradigms assessing spatial and reversal learning, as well as object recognition memory were used to evaluate cognitive capabilities in

Cacna1c$^{+/-}$ compared to *Cacna1c*$^{+/+}$ rats. Results show that neither *Cacna1c*$^{+/-}$ nor *Cacna1c*$^{+/+}$ rats exhibited strong cognitive impairments and in fact, *Cacna1c* haploinsufficient rats appear to show normal, and in some cases above normal, spatial learning over a seven-day period. However, during reversal learning male *Cacna1c*$^{+/-}$ rats displayed reduced cognitive flexibility. In terms of cognitive flexibility in *Cacna1c*$^{+/-}$ female rats, they showed an ideal behavior pattern entering the newly baited arms above chance, the never baited arms below chance, and previously baited at chance levels. *Cacna1c*$^{+/+}$ females on the other hand, still tended to enter previously baited arms at above chance levels. Interestingly, in males this relationship is almost the opposite, this may point towards differential mechanisms in how $Ca_v1.2$ expression levels affect cognition and behavior in the two sexes. *Cacna1c*$^{+/+}$ females, however, over the course of reversal learning were able to adapt their behavior to the new configuration and, thus, became similar in comparison to their *Cacna1c*$^{+/-}$ littermates. In novel object recognition, irrespective of genotype, males and females performed equally as well and were successfully able to distinguish between the novel and familiar object. Taken together, the results from Study III suggest that in *Cacna1c* rats spatial memory and reversal learning capabilities show initial impairments in cognitive flexibility in *Cacna1c*$^{+/-}$ males but better long-term learning and initial hypo-activity at a slightly better performance in *Cacna1c*$^{+/-}$ females. Novel object memory, though, appears intact. It therefore, appears that *Cacna1c* haploinsufficiency has a slight positive impact on spatial memory abilities in rats.

Review I

Title: From play to aggression: High-frequency 50-kHz ultrasonic vocalizations as play and appeasement signals in rats

Authors: Theresa M. Kisko, Markus Wöhr, Vivien C. Pellis and Sergio M. Pellis

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From Play to Aggression: High-Frequency 50-kHz Ultrasonic Vocalizations as Play and Appeasement Signals in Rats

Theresa M. Kisko, Markus Wöhr, Vivien C. Pellis and Sergio M. Pellis

Abstract When rats engage in playful interactions, they emit appetitive 50-kHz ultrasonic vocalizations (USVs). We investigated the role of 50-kHz USVs in the playful behavior of both juvenile and adult rats. A cohort of juvenile rats was surgically devocalized and allowed to interact with either devocalized or intact partners as juveniles and again as adults. A substantial decrease in playful motivation was seen for pairs of devocalized rats, as well as all intact rats housed with devocalized ones. In pairs in which at least one partner could vocalize, there was no difference in the number of playful interactions as compared to controls. Further investigation revealed that, within the playful episode itself, 50-kHz USVs are more likely to appear before a playful attack is launched than after, regardless of the attacking partner's ability to vocalize, and when one partner is pinned on its back by another, it is the rat that is on top that is more likely to emit 50-kHz USVs. These findings suggest that, for juveniles, 50-kHz USVs may have a critical function in maintaining and facilitating playful motivation, but a more limited role in signaling playful actions. In adults, however, whatever the motivational role of such calling may be, the various kinds of USVs appear to serve critical communicatory functions. For instance, when pairs of adult males that are unfamiliar with one another encounter each other in a neutral arena, they play together, but if one partner is devocalized, there is a significantly higher likelihood that the interaction will escalate to become aggressive. While the relative roles of appetitive 50-kHz and aversive 22-kHz USVs in this context remain to be determined, our overall findings for play in both juveniles and adults suggest that 50-kHz USVs likely have multiple functions, with different functions being more prevalent at some ages and contexts than others.

T.M. Kisko (✉) · M. Wöhr
Behavioral Neuroscience, Experimental and Biological Psychology,
Philipps-University of Marburg, Gutenbergstr. 18, D-35032, Marburg, Germany
e-mail: theresa.kisko@gmail.com

V.C. Pellis · S.M. Pellis
Canadian Centre for Behavioural Neuroscience, Department of Neuroscience,
University of Lethbridge, Lethbridge, AB T1K 3M4, Canada

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Keywords Rough-and-tumble play · Rats · Play signal · Appeasement signal · Ultrasonic vocalizations

Contents

1 Introduction

One of the most challenging forms of play is rough-and-tumble play or play-fighting, in which pairs of animals typically compete for some advantage over one another (Aldis 1975). This advantage, often involving contacting some target on the body (Aldis 1975; Pellis 1988), does not involve the unrestrained competition often seen in serious fighting (Blanchard and Blanchard 1994), but rather involves some measure of restraint that leads to play-fighting being reciprocal. Such reciprocity has been characterized by the 50:50 rule, whereby each player wins about 50 % of its playful encounters (Altmann 1962). Subsequent game theory models have shown that, as win–loss ratios deviate from 50:50, play-fights become progressively less stable. This is not to say that in some cases, the win–loss ratio cannot deviate from 50:50 (for review see Pellis et al. 2010), but what it does suggest is that play-fighting will not remain playful if one partner attempts to dominate the encounters completely. Indeed, some empirical studies have shown that, when individuals do attempt to dominate playful interactions completely, their potential play partners ostracize them, reducing their ability to engage in further interactions (e.g., Suomi 2005).

To maintain the reciprocity needed for play-fights to remain playful, animals have to follow rules of restraint (Pellis et al. 2010), which requires them to monitor both their own actions and those of their partner, to evaluate any potential transgressions of the rules. To be precise, this requires that, while engaged in play, animals need to assess whether an inappropriate action by a partner is a one-off act of exuberance or a deliberate bending of the rules. The possibility of deliberate transgressions becomes particularly likely as animals become sexually mature and increasingly use play-fighting as a tool for social assessment and manipulation (Palagi 2011). Indeed, comparative data suggest that social play is a more

demanding activity than nonsocial play. Comparative analyses in primates have shown that the size of socioemotional brain systems increases in species that engage in more play-fighting, but not in species that engage in more nonsocial play (i.e., locomotor-rotational play, object play) (Graham 2011). One of the mechanisms thought to be involved in maintaining play-fighting, not only in primates but also in other species, is the use of play signals to negotiate interactions (Palagi et al. 2015). Rats not only engage in complex patterns of play-fighting, but they also use signals that can potentially serve the negotiating functions needed to maintain playfulness (Pellis and Pellis 2009).

2 Play Behavior in Rats

Rats engage in a variety of forms of play, including playing with inanimate objects, solitary locomotor-rotational play, and play-fighting (Hole and Einon 1984). However, even though locomotor-rotational play and play-fighting in rats can be very complex, object play is limited, and by far, it is play-fighting that occupies the majority of their time when they are playing (see Pellis and Pellis 2009 for a review). Not surprisingly, the rat has been an important model species for the study of the behavioral, developmental, and neurobiological mechanisms underlying mammalian play-fighting (e.g., Siviy and Panksepp 2011; Vanderschuren and Trezza 2014).

In rats, play-fighting involves attack and defense of the nape of the neck, which is then nuzzled with the snout when contacted (Pellis and Pellis 1987; Siviy and Panksepp 1987). Such dorsal contact by one partner is defended against by the recipient by using one of two major classes of defensive tactics: (1) evasion, in which the rat turns to look away from the oncoming attacker and swerves, leaps or runs away, and (2) facing defense, in which the rat turns to face the attacker and uses a variety of movements to block access to its nape. Facing defense, in turn, can involve two different classes of tactical maneuvers: (1) rotation around a vertical axis, usually the mid-body or pelvis, thus maintaining a prone position, and (2) rotation around the longitudinal axis, with either the whole body rotating so that the defender lays supine on its back or with only the forequarters rotating so that the defender still maintains contact on the ground with at least one hind foot (Himmler et al. 2013). If successfully executed, the defender can then launch counterattacks of its own, which, if successful, can lead to a role reversal as the original attacker defends itself (Kisko et al. 2015a). Moreover, the attacker may execute movements that facilitate successful counterattacks by the defender (Pellis et al. 2005), thus ensuring reciprocity. Regardless of the pattern of defense used, the repeated attacks and defense often lead to one rat lying on its back and its partner standing over it in a pinning configuration (Panksepp 1981).

Due to the repeated cycles of attack, defense, and counterattack, play-fighting in rats is thought to be more complex than that reported in many other species of rodents (Pellis and Pellis 1998) and as complex as that reported in many species of

primates and carnivores (Pellis and Pellis 2009). Consequently, play-fighting in rats, like in many primates (Palagi 2011), involves complex cognitive assessments and the regulation of emotions (Pellis et al. 2014). To maintain such complex processes and thus allow playful interactions to proceed, rats likely depend on the use of play signals.

3 Play Signals

Because the contact involved in play-fighting can be similar to that occurring in other social contexts, such as aggression and courtship, it has been hypothesized that animals can use play signals to inform their partners that the contact is playful (Fagen 1981). While play signals can be used to make amends if one animal is too rough in its actions (Aldis 1975), the traditional role of such play signals has been thought to be to inform a potential play partner that the imminent contact is playful (Bekoff 1975). Play signals can be produced in several sensory modalities, including olfactory (Wilson 1973) and auditory ones (Kipper and Todt 2002), but ones involving visual cues are the most widely reported (Palagi et al. 2015). Among canids and primates, facial gestures provide the richest source of signaling (Bekoff 1975; van Hoof 1967), but bodily movements and positions are also prevalent (Yanagi and Berman 2014). In rats, facial gestures are limited to basic ones exhibiting pleasure and revulsion (Berridge and Robinson 2003), and there is no evidence of olfactory signals being used in play (Hole and Einon 1984). Rats have a rich repertoire of jumps, twists, turns, and runs that are performed during playful interactions (Pellis and Pellis 1983), and these could potentially serve as play signals. Other rodents with complex playful wrestling, such as hamsters, do not have these bodily gyrations (Pellis and Pellis 1988), yet mice that do not engage in playful wrestling have a varied repertoire of jumps and rotations (van Oortmerssen 1971). Therefore, it is unlikely that *all* these bodily gestures function as play signals. Nonetheless, some of these jumps performed by rats are produced in contexts that are consistent with them being useful for facilitating play (Pellis and Pellis 1983); this suggests that some may serve as communicatory functions. More likely to function as play signals, however, are the rich diversity of ultrasonic vocalizations (USVs) that are emitted in a variety of prosocial contexts, including play-fighting (Burgdorf et al. 2008; Knutson et al. 1998; Wright et al. 2010).

4 Ultrasonic Vocalizations in Rats

Rats are able to emit sounds in the ultrasonic range, termed USVs. Typically, three main categories of USVs are distinguished, of which all serve distinct communicative functions as socioaffective signals (for a detailed overview, see Brudzynski 2013; Wöhr and Schwarting 2013). Infant rats emit 40-kHz USVs following

separation from their mother and littermates. These 40-kHz USVs elicit maternal behaviors, most notably, search and retrieval behavior (Wöhr and Schwarting 2008). In juvenile and adult rats, two main USV types occur, with their occurrence strongly depending on the emotional valence of the situation. Low-frequency 22-kHz USVs occur in aversive situations and particularly high rates are observed during aggressive encounters of adult male rats (Lehman and Adams 1976; Lore et al. 1976; Sales 1972b; Sewell 1967). They likely reflect a negative affective state. In contrast, high-frequency 50-kHz USVs are observed in appetitive situations, most notably in juveniles both during rough-and-tumble play with peers (Burgdorf et al. 2008; Knutson et al. 1998; Wright et al. 2010) and when tickled by a human (Panksepp and Burgdorf 2000). However, some negative affective situations, such as resident–intruder tests, will also elicit 50-kHz USVs (Takahashi et al. 1983). In adulthood, 50-kHz USVs mainly occur during mating (Sales 1972a), but can also be seen in other rewarding situations, such as when given food (Burgdorf et al. 2000) and psychoactive drugs (Burgdorf et al. 2001, 2008). It is widely believed that they reflect a positive affective state. In the first study on 50-kHz USVs emitted during rough-and-tumble play, Knutson et al. (1998) showed that the emission of 50-kHz USVs is positively correlated with dorsal contacts during play and that 50-kHz USVs occur in anticipation of play. As described by Wöhr et al. (2015), they further found that rats exposed to a brief period of social isolation emitted more than twice as many 50-kHz USVs and that they played more vigorously than group-housed controls, possibly due to an increase in social motivation. In contrast, an aversive stimulus, such as a bright white light, led to a reduction in 50-kHz USV emission. In a subsequent study, Burgdorf et al. (2008) found that, of the many 50-kHz USV subtypes, the frequency-modulated (FM) 50-kHz USVs occur at particularly high rates during rough-and-tumble play. These subtypes are also greatly increased following a brief period of social isolation and are most closely associated with the occurrence of dorsal contacts during play, but are negatively correlated with pinning behavior. Interestingly, in rats selectively bred for low 50-kHz USV emission rates, rough-and-tumble play is altered and characterized by fewer dorsal contacts but more pinning behavior (Webber et al. 2012). Moreover, in rats selectively bred for low or high anxiety-related behavior, we found that highly anxious rats initiate less rough-and-tumble play and emit fewer 50-kHz USVs, possibly reflecting lack of positive affect (Lukas and Wöhr 2015). As the breeding lines differ in their hypothalamic vasopressin availability and vasopressin is strongly implicated in the regulation of social behavior, we further tested whether manipulating the vasopressin system alters the emission of 50-kHz USVs during rough-and-tumble play. While the administration of synthetic vasopressin did not alter rough-and-tumble play and the concomitant emission of 50-kHz USVs, blocking the central vasopressin system by means of a vasopressin 1a receptor antagonist resulted in lower levels of play behavior and fewer 50-kHz USVs (Lukas and Wöhr 2015). This indicates that the central vasopressin system is involved in the regulation of affiliative communication in rodents, which is of translational relevance because various findings repeatedly link alterations in the central vasopressin system to autism in humans. Recently, we further showed that rats exposed

to valproic acid during pregnancy emit fewer 50-kHz USV during rough-and-tumble play (Raza et al. 2015). Exposure to valproic acid, which is a drug typically used to treat epilepsy and bipolar disorder, is one of the major environmental risk factors for developing autism in humans (Moore et al. 2000) and has been shown to induce autism-like phenotypes when administered to pregnant rats (Schneider and Przewłocki 2005).

5 High-Frequency 50-kHz USV as Play Signals?

The close relationship between the play behavior and the emission of 50-kHz USVs suggests that 50-kHz USVs might serve a communicative function as play signals. If 50-kHz USVs are being used as traditional play signals, signifying "I want to play with you" (Bekoff 1975), then the most important characteristic would be that they occur most frequently preceding playful attacks. In a recent study (Himmler et al. 2014), we provided support for such use of 50-kHz USVs in juvenile rats. We showed that there were significantly more 50-kHz USVs emitted preceding playful contact compared to when rats cease contact. We also showed that, consistent with other studies (Lukas and Wöhr 2015), 50-kHz USVs occur more often in males than in females during play-fighting. This sex difference may be associated with the play of males tending to be rougher (Pellis et al. 1997). Rougher play poses a bigger threat in escalating to serious aggression and so may be more reliant on play signals to avoid such escalation (Palagi et al. 2015). Furthermore, because of the variety of 50-kHz USVs, we also explored whether particular 50-kHz USV subtypes are associated with the onset of specific defense tactics. In the Himmler et al. (2014) study, the most frequently emitted 50-kHz USV subtype was the trill, but this subtype was not significantly associated with any specific defensive action. Short calls, although less frequent, mainly occurred when the defender used an evasive tactic. These findings, especially those showing the high frequency of calling preceding contact, provide compelling evidence supporting the traditional function of play signals, that of advertising imminent contact of one partner by another (Bekoff 1975). In this study, however, both rats could vocalize, so any particular call could not be empirically attributed to either partner. Therefore, it cannot be certain whether the rat launching the attack was in fact the one vocalizing prior to making contact.

A procedure to overcome this dilemma is surgical devocalization, which has been previously used to study the communicative function of USVs in adult rats (Lehman and Adams 1976; Takahashi et al. 1983; Takeuchi and Kawashima 1986; Thomas et al. 1983). Therefore, using pairs of juvenile rats in which one partner was vocal and the other devocalized, we examined which partner, prior to a playful attack, was vocalizing (Kisko et al. 2015a). It was predicted that, when a devocalized rat attacks a vocal partner, there should be very few, if any, 50-kHz USVs being emitted prior to that attack. However, this was not the case, and, in fact, the number of 50-kHz USVs emitted prior to an attack when the devocalized partner

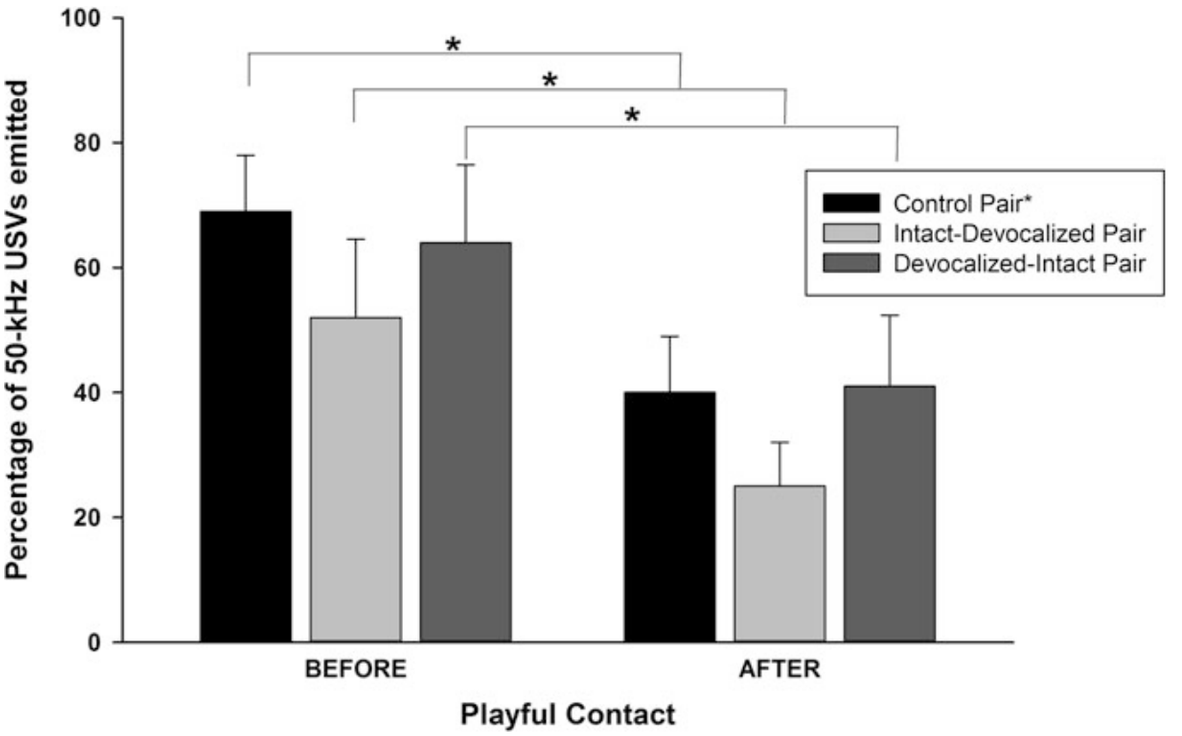

Fig. 1 Percentage (mean and SEM) of 50-kHz USVs emitted immediately before playful contact and immediately following the termination of contact. More 50-kHz USVs are emitted prior to contact whether the attacker is the one able to vocalize or not (*$p < 0.05$; the control pair is from Himmler et al. 2014; the graph is a combined data set from Himmler et al. 2014 and Kisko et al. 2015a)

attacked was comparable to the number of 50-kHz USVs emitted when a vocal rat was attacking. That is, the same pattern (Fig. 1) that was found whether both partners could vocalize (Himmler et al. 2014) or only one could do so (Kisko et al. 2015a). Moreover, we found no difference in the subtypes of 50-kHz USV emitted, irrespective of which partner was attacking (Kisko et al. 2015a). These findings suggest that the rats are not only using 50-kHz USVs to announce an attack but also to solicit playful contact from a partner.

Tickling juvenile rats by a human experimenter elicits high rates of 50-kHz USVs (Panksepp and Burgdorf 2000); this action is thought to mimic rough-and-tumble play between two rats. In particular, when tickled, rats roll over onto their backs, thus adopting a configuration similar to that of the pinning present in play-fighting. This suggests that rats produce many calls while on their backs. If this were the case, it would seem reasonable that many, if not the majority of 50-kHz USVs emitted during play-fights, should be emitted by the rat that is being pinned.

Contrary to expectation, data analyzed from our pairs of rats in which one partner was devocalized (Kisko et al. 2015a) revealed that more 50-kHz USVs occurred when the vocal rat was pinning the devocalized rat than when the devocalized rat was pinning the vocal rat (Fig. 2). However, given that the rate of pinning by devocalized rats was low, data based on six pairs of rats, even though significant, should be considered preliminary. If substantiated by further studies, these observations would suggest that it is not the tickling of the belly itself that

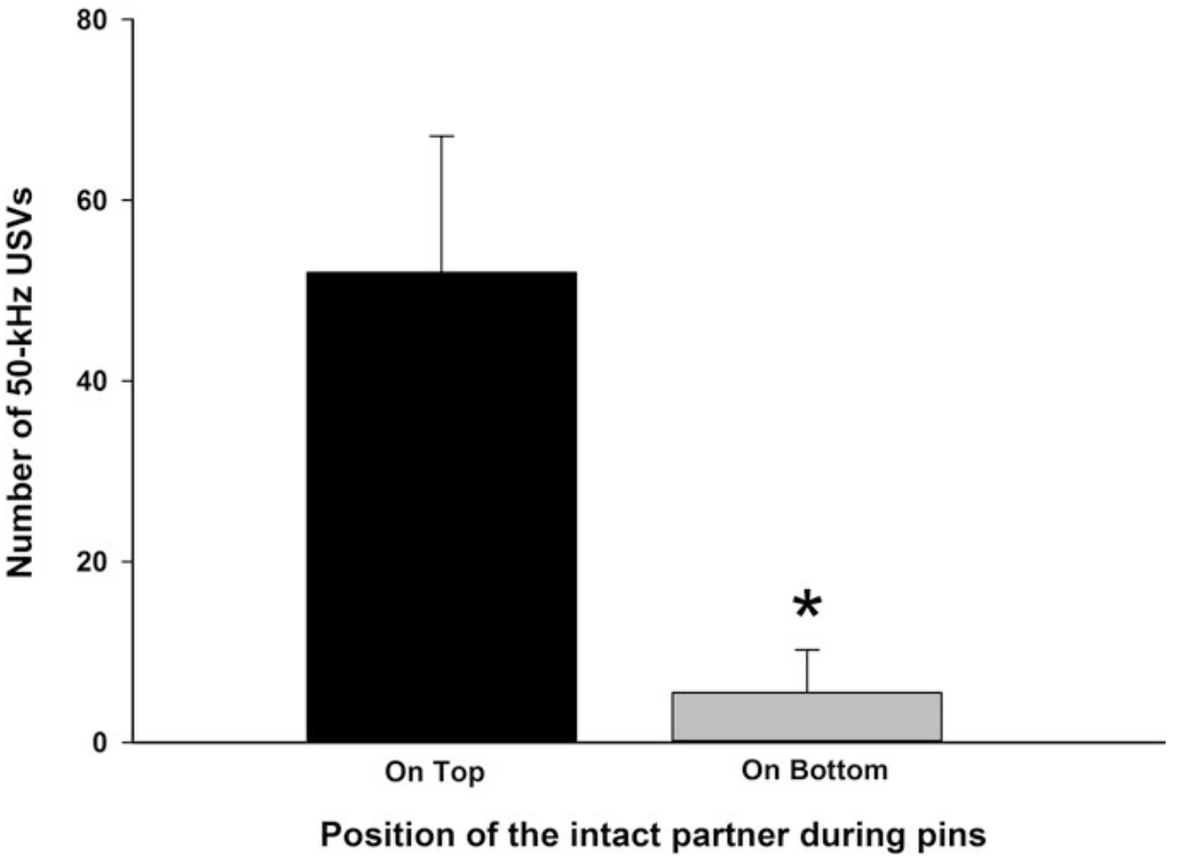

Fig. 2 Frequency of occurrence of 50-kHz USVs (mean and SEM) when rats are pinned during playful interactions. 50-kHz USVs are more frequent when the intact rat is on *top* than when it is on the *bottom* (*$p < 0.05$)

elicits 50-kHz USVs during play-fighting, but rather, it is the rat on top—the "tickler"—that receives the most enjoyment, thus emitting more 50-kHz USVs. Some level of resistance by the partner being attacked seems to be critical in motivating playful attacks (Pellis and McKenna 1995), so that initiating, soliciting, and gaining contacts together ensure rewarding tactile experiences during play. The presence of 50-kHz USVs in all these phases of play-fighting provides support for the hypothesis that 50-kHz USVs express the rats' positive affective state and so function to maintain the animals' playful motivation.

In further support of the hypothesis that juvenile rats are using 50-kHz USVs to keep the mood playful and in doing so maintain playful interactions, we found that pairs of devocalized rats had a reduced frequency of playful interactions (Kisko et al. 2015a). When compared to pairs of vocal rats, devocalized pairs had almost 50 % fewer play-fights (Fig. 3). This suggests that 50-kHz USVs are being used to promote and maintain a playful mood and, in their absence, the rats are not nearly as motivated to engage in play. It is possible that this playful mood is linked to dopamine. Studies have shown that play-fighting is associated with the release of dopamine in the nucleus accumbens (Trezza et al. 2010) and that activation of the mesolimbic dopamine system induces the production of 50-kHz USVs (Burgdorf et al. 2001, 2007). Using the playback paradigm, we found that hearing 50-kHz

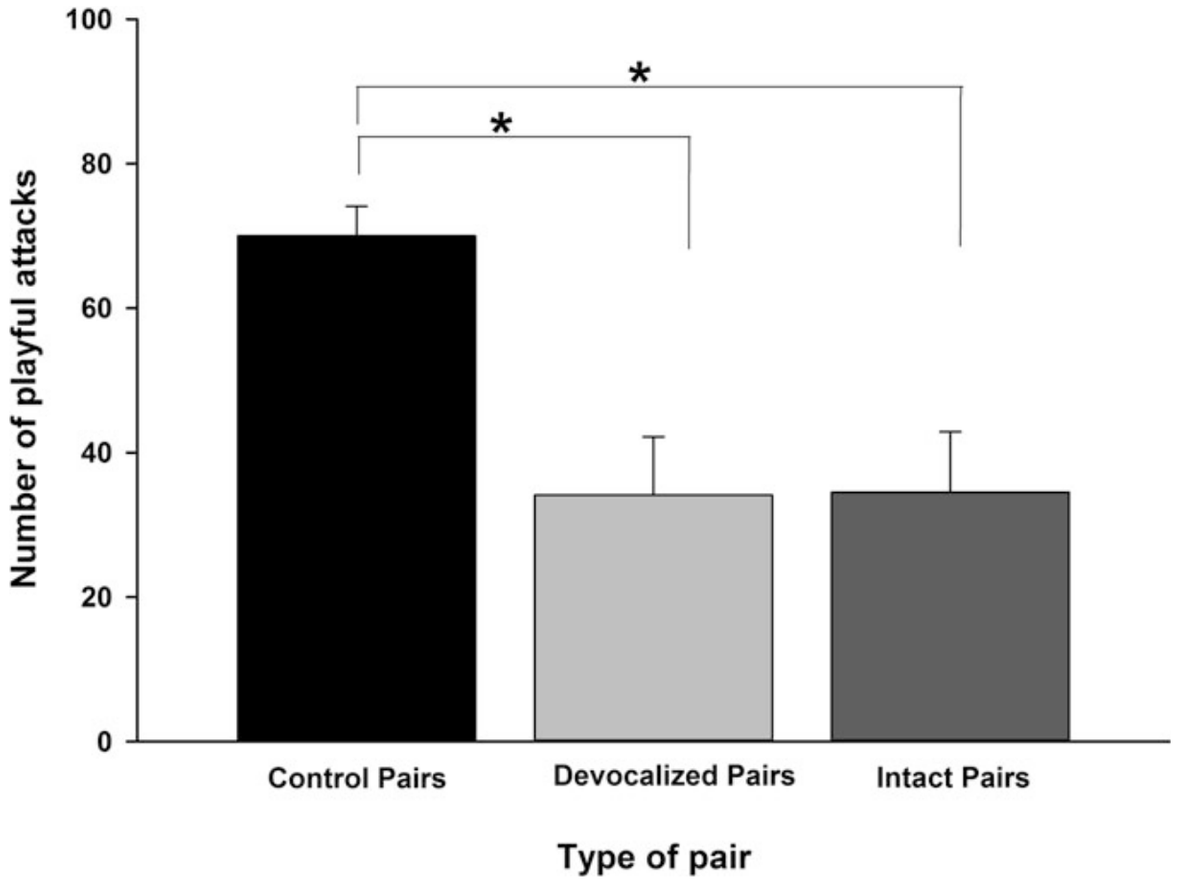

Fig. 3 Number of playful attacks (mean and SEM) initiated by pairs of intact rats reared with other intact rats (control pairs), by pairs of devocalized rats (devocalized pairs), and by pairs of intact rats reared with devocalized partners (intact pairs) in 10-min trials. Both the devocalized rats and the intact cage mates of devocalized rats exhibit a reduced motivation to engage in play (*$p < 0.05$)

USVs results in increased neuronal activity (Sadananda et al. 2008) and dopamine release (Willuhn et al. 2014) in the nucleus accumbens. This suggests that the release of dopamine in the nucleus accumbens is linked to both the production and the perception of 50-kHz USVs, possibly indicating that dopamine release in the nucleus accumbens functions as a translator of a motivational acoustic signal into a prosocial action. Such a perception-and-action loop is particularly relevant for appetitive social and reciprocal communicatory signals, with 50-kHz USVs reflecting a positive affective state in the sender and evoking a similar affective state in the receiver, thus promoting positive social interactions.

Interestingly, the playful mood can be reinstated to the typical control levels, seen in pairs of vocal rats, by pairing a devocalized rat with an unfamiliar vocal partner (Kisko et al. 2015b). This provides further support for the motivational role of 50-kHz USVs. The motivational role of 50-kHz USVs, however, may have a critical learning period. We observed that, in juveniles, the overall playful motivation was not only decreased in pairs of devocalized rats but was also significantly decreased in pairs of vocal rats that were housed with devocalized cage mates (Fig. 3). Juvenile cage mates often engage in playful interactions together, and it is

possible that, in this critical learning period for juveniles, a vocal rat playing with a devocalized cage mate may not receive the necessary feedback from hearing 50-kHz USVs to learn about their contextual uses. That is not to say that the calls themselves are learned, but rather, that the proper context for their use in some situations could be learned through play (see, for further evidence, Wöhr et al. 2015). The animals in our study were housed in quads of two devocalized and two intact rats, so one would think that when the vocal cage mates played together it would be sufficient for them to learn the contextual cues, but this does not appear to be the case. In support of this critical learning period for juveniles, we have recently shown that prolonged social isolation in the four weeks after weaning, the juvenile period when play-fighting is most frequent results in a lack of appropriate behavioral responses toward 50-kHz USVs (Seffer et al. 2015). Specifically, while group-housed controls displayed social approach behavior in response to 50-kHz USVs, a response that is even more prominent in rats isolated for 24 h, rats exposed to long-term, post-weaning, social isolation did not display social approach behavior. Furthermore, these rats even showed some signs of social avoidance. In contrast, no social deficits were seen in rats given comparable levels of long-term social isolation following the juvenile period. Juvenile rats socially isolated for 24 h have an increased motivation to engage in playful interactions (Himmler et al. 2013); however, this increase can be curtailed by placing them with less playful partner. For example, a partner treated with scopolamine, a cholinergic antagonist, will explore the enclosure in which it is placed, but will not initiate playful attacks or respond to a playful attack (Pellis and McKenna 1995). Such a partner elicits playful attacks initially, but prolonged exposure to such a partner leads to reduced playful motivation in the un-drugged animal, as evidenced by a decrease in initiating playful attacks (Pellis and McKenna 1995). Furthermore, social play generally occurs only when a rat is free from physiological and social stress (Siviy et al. 2006). The decreased motivation to play that is seen in the devocalized cage mates could, in turn, negatively impact the playful motivation of the vocal cage mates. As a result, if a lack of playful motivation is consistent and prolonged, as would be the case for the vocal cage mates of the devocalized rats, the vocal rats may become depressed or stressed and thus much less motivated to play.

As well as regulating playful mood, 50-kHz USVs may also serve other important communicatory functions. For rats, pinning and being pinned during play-fighting appears to be highly rewarding and is thus a substantial component within their playful repertoire (Panksepp 1981). In a study by Siviy and Panksepp (1987), it was found that deafened rats pinned less, suggesting that not being able to hear 50-kHz USVs decreases the desire for close bodily contact in playful situations. Similarly, it was hypothesized that devocalized pairs would also show a reduction in playful pinning defenses, but the opposite turned out to be true (Kisko et al. 2015a). Pairs of devocalized rats had a higher frequency and preference for contact-promoting playful defenses than the intact control pairs. One hypothesis to explain these results could be that the 50-kHz USVs are acting as contact calls to help localize the partner within the play arena. Being nocturnal, the majority of playful interactions in rats take place in the dark, and so, being able to signal their

location to their partner in a non-visual manner would be beneficial. If this were so, this could explain why devocalized pairs prefer to stay in close contact, in that it would avoid spending long amounts of time searching for one another in a test arena. Therefore, we predicted that, if a vocal rat were paired with a devocalized partner, the devocalized rat would adopt the typical playful defense tactics seen in control rat pairs, since calls from the vocal rat would provide the means to locate that partner. That is, by being able to hear their play partner's 50-kHz USVs and adopting the more typical tactics of defense, the devocalized rats would return to the pinning frequencies present in control pairs. However, even when paired with a vocal partner, the devocalized animals still appeared to prefer to use contact-promoting defense tactics significantly more often than evasive defense tactics. This suggests that the change in defensive actions by the devocalized rats is not to compensate for the absence of 50-kHz USVs. Therefore, at least within the confines of the test arena used in this study, the results do not support the contact call hypothesis.

Moreover, when given the choice of being presented in the same test arena, vocal rats are no more attractive as a play partner than are silent ones (Kisko et al. 2015a). Indeed, even when confronted with unfamiliar animals, the rats were just as likely to launch playful attacks on devocalized partners as they were on vocal ones (Kisko et al. 2015b). That is to say, among juveniles, there is little evidence that rats use 50-kHz USVs as traditionally conceived play signals (Bekoff 1975; Palagi et al. 2015)—they appear to be unnecessary for both initiating playful contact and in soliciting playful contact. That for juvenile rats 50-kHz USVs do not appear to provide rewarding social incentives (Willey and Spear 2012) is consistent with these findings (although see below). Rather, the role of 50-USVs seems more closely tied to regulating playful motivation and possibly in promoting the development of prosocial neural systems.

A commonly used measure of playful motivation is the frequency with which rats initiate playful contacts on the nape of their partner (Himmler et al. 2013). Such attacks are diminished when pairs of devocalized rats are tested together (Kisko et al. 2015a). Moreover, role reversals, in which the original defender launches a successful counterattack, forcing the original attacker to defend itself, are also reduced in such pairs (Kisko et al. 2015a). Given that the frequency of such counterattacks are decreased in tandem with initiating attacks (Pellis and Pellis 1990), the reduced frequency of role reversals is also likely to reflect a reduction in the motivation to play. That these reductions are, at least in part, due to an acute effect of the absence of 50-kHz USVs on playful motivation is suggested by the restoration of a high frequency of playful attacks when devocalized rats are tested with unfamiliar, vocal partners (Kisko et al. 2015b). However, that some of this effect is due to a more chronic influence of lack of exposure to normal levels of 50-kHz USVs over a prolonged period is shown by the finding that the vocal partners of devocalized cage mates also show a depressed level of initiating playful attacks (Fig. 3). In addition, the altered pattern of playful defense present in devocalized rats (Kisko et al. 2015a) is not ameliorated when playing with an unfamiliar, vocal partner (Kisko et al. 2015b), further suggesting deeper

organizational changes in brain development due to a chronic lack of vocalizing. All our devocalized rats received their surgeries at around postnatal day 25, an age within a critical period for the development of several neurotransmitter and neuropeptide systems implicated in the regulation of social behavior (Trezza et al. 2010). The observation that the rats with sham surgeries did not display the same changes in play-fighting implicates the role of cutting laryngeal nerves, and the associated elimination of the ability to produce 50 or 22-kHz USVs, in these developmental disturbances.

Unlike the study by Wiley and Spear (2012), some playback studies have shown that 50-kHz USVs do appear to provide rewarding social incentives. For instance, Burgdorf et al. (2008) found that rats will nose-poke to elicit playback of 50-kHz USVs. Moreover, we showed that playback of 50-kHz USVs results in social approach behavior in the recipient (Wöhr and Schwarting 2007; Willuhn et al. 2014), and as described by Wöhr et al. (2015), this response is present in both juveniles and adults. However, as already mentioned, long-term, post-weaning social isolation results in a lack of social approach behavior in response to 50-kHz USVs (Seffer et al. 2015). These latter findings are consistent with the notion that the juvenile period is an important one for the development of the neural systems associated with 50-kHz USV production. Thus, given the possibility that the neural systems associated with the production of USVs and those associated with the regulation of social behavior overlap in their development, the changes in social play wrought by chronic devocalization in the early juvenile period that we have found (Kisko et al. 2015a, b) may not be coincidental. Such effects may be used as a vehicle for exploring how these neural systems may interact.

6 High-Frequency 50-kHz USVs as Appeasement Signals?

As noted above, for play-fighting to remain playful, the participants need to exercise some degree of reciprocity. Transgressions can lead to the partner escalating the encounter into serious aggression. Among juveniles, such escalation is rare, but not absent (Fagen 1981). It has been suggested that play signals can be used in such situations to de-escalate the encounter with the transgressor effectively using the signal to inform the partner that "it was only play" (Aldis 1975). That is, the signal can be used to appease the partner. In rats, play-fighting can also occasionally escalate into serious fighting, which can be unambiguously identified as when the rats stop attempting to nuzzle each others' napes and instead switch to bite the partner's lower flanks and rump (Pellis and Pellis 1987, 1990). If 50-kHz USVs are used as signals to de-escalate the risk of a playful encounter turning into aggression, then, in the absence of these calls, such escalation should be more likely. For none of our juvenile experimental animals were play-fights found to escalate into aggression—not when devocalized rats played together or when devocalized rats played with vocal partners (Kisko et al. 2015a). Even when tested with unfamiliar partners, so eliminating the possibility that rats with an established

relationship can use other means to avoid escalation, there was no evidence that play-fights were more likely to escalate to aggression when one of the rats could not vocalize (Kisko et al. 2015b). The situation appears to be different when adult rats are involved.

In some species, adults also engage in play-fights, at which age it is likely to be used for social assessment and manipulation (Palagi 2011). Among adult male rats, dominance relationships can be negotiated with play-fights (Pellis and Pellis 2009). Within colonies of familiar rats, subordinate males will initiate and engage in a more gentle form of play with a dominant male. Furthermore, they will initiate less play with other subordinates, and when they do play together, it will be rougher. When unfamiliar adult rats encounter one another in a neutral arena, they can engage in a rough form of play-fighting which can lead to the establishment of a dominance relationship. When neither member of a pair adopts a submissive status, the encounter can escalate into serious fighting (reviewed in Pellis and Pellis 2009). It is hypothesized that, if 50-kHz USVs serve an important communicative function as appeasement signals, then this should become apparent when unfamiliar, adult males encounter one another in a neutral arena.

In pairs in which one play partner is devocalized, the risk of the interaction becoming aggressive is significantly higher than in pairs in which both rats can vocalize (Kisko et al. 2015b). In fact, in all pairs that included an unfamiliar devocalized partner, there were both agonistic displays, such as piloerection, lateral displays, and tail wiggles, and aggressive attacks, in which one partner directs bites at the flanks of the opponent. Such agonism was rare in the pairs in which both rats could vocalize, and their encounters never escalated to biting. This strongly indicates that, in potentially risky and ambiguous situations, adults may rely on 50-kHz USVs to modify each other's behavior tactically in a way that is not essential among juveniles. These findings are thus consistent with ones that show that 50-kHz USVs are used as signals in agonistic encounters in adult rats.

In resident–intruder tests, in which an unfamiliar adult male is placed in the home cage of a resident male, the resident typically attacks the intruder (Blanchard and Blanchard 1994), and in such encounters, 50-kHz USVs are frequently emitted (Sales 1972b; Sewell 1967). Moreover, rats are even found to emit 50-kHz USVs when entering an area associated with the potential presence of an aggressor, with the number of 50-kHz USVs emitted by the intruder being positively correlated with the number of aggressive encounters it has experienced in this enclosure (Tornatzky et al. 1994, 1995). Importantly, devocalization (Takahashi et al. 1983; Thomas et al. 1983) and pharmacological (Vivian and Miczek 1993) studies have implicated the intruder as the source of the 50-kHz USVs. Together, these findings indicate that 50-kHz USVs are emitted as a signal of appeasement, thus reducing the likelihood of being attacked by the resident.

It should be noted that devocalization abolishes not only the ability to produce 50-kHz USVs, but also 22-kHz USVs, and there is evidence for 22-kHz USVs being used as an appeasement signal, but results are conflicting. For instance, it was reported that in the resident–intruder paradigm, aggressive behavior is rarely observed following the emission of 22-kHz USVs (Lehman and Adams 1976;

Lore et al. 1976; Sales 1972b; Sewell 1967); yet devocalization experiments do not support the idea that 22-kHz USV emission modulates the aggressive behavior of the resident (Lehman and Adams 1976; Takeuchi and Kawashima 1986; Thomas et al. 1983). Thus, in the neutral test arena that we used (Kisko et al. 2015b), either 50-kHz USVs alone, 22-kHz USVs alone, or some combination of both may be used to diminish the likelihood of escalation from playful to serious fighting.

7 Conclusion

50-kHz USVs are emitted at a high frequency during play-fighting among juvenile rats (Burgdorf et al. 2008; Knutson et al. 1998; Wright et al. 2010). Examination of when these calls occur during play-fights shows that they are most likely to occur immediately prior to playful contact (Himmler et al. 2014), and this is true whether the attacker can vocalize or not (Kisko et al. 2015a). These findings suggest that the production of USVs is integral to play and that they may provide important communicatory functions. Such vocalizations may serve two kinds of communicatory functions that have been traditionally postulated for play signals (Bekoff 1975; Palagi et al. 2015): that of informing a potential recipient of a playful attack and that the imminent contact will be playful or that of a recipient soliciting such an attack from a nearby partner. However, our findings with devocalized rats indicate that play can occur in the absence of these presumed communicatory functions, at least among juveniles (Kisko et al. 2015a, b). More consistent with our data is the hypothesis advocated by Knutson et al. (1998) and supported by others that 50-kHz USVs are an expression of the positive affective state associated with play (Burgdorf et al. 2008). In this context, if these calls do serve a communicatory role, it is an indirect one, that of maintaining the playful mood of the producer and/or the receiver of the calls (Kisko et al. 2015a, b). From a developmental perspective, the production and perception of such calls may be important for the maturation of neural and behavioral systems that are associated with prosocial behavior. The case for a communicatory role of USVs is more compelling for adult males engaging in playful interactions with unfamiliar partners. In the absence of such calling, even when just one member of the pair cannot vocalize, there is a marked increase in the likelihood that play-fights escalate into aggression. Given the evidence from resident–intruder encounters (e.g., Lore et al. 1976; Sales 1972b), it seems highly likely that USVs, be they 50-kHz USVs, 22-kHz USVs, or both, are being used as appeasement signals to attenuate the risk of playful encounters escalating into aggression (Kisko et al. 2015b). Therefore, it seems that 50-kHz USVs may have multiple functions, and these may differ across different stages of development.

Further developmental studies are needed to understand the roles of maturation and learning in being able to emit 50-kHz USVs in contextually relevant ways during social interactions. Surgical devocalization provides a clear way to examine the role of such vocalizations and enables observers to identify, unambiguously, the rat that is vocalizing when only one member of a dyad is devocalized (Kisko et al.

2015a, b; Thomas et al. 1983). However, the technique is highly invasive and may have potential long-term side effects that have not yet been investigated. Therefore, other methods that do not require surgery to identify which member of the pair is calling would be helpful. One such technique, the use of multiple microphone arrays, has been successfully used to examine the emission of USVs during mating and other social interactions in mice (Neunuebel et al. 2015). However, play-fighting in rats is often very vigorous and fast-paced, with the rats alternating between wrestling and running. While worth trying, it seems unlikely that the multiple microphone array technique would be able to distinguish which rat is calling in all situations during play. Another new and potentially useful technique is one being used in songbirds, in which an ultraminiature backpack is used to record sound and acceleration in the bird carrying the pack (Anisimov et al. 2014). The small backpack, which weighs only 2.6 g, is harnessed on the bird's back. Moreover, the weight of the backpack can be further decreased, to 1.4 g, if necessary. This backpack monitoring system may be an ideal way to record the vocalizations of individual rats or mice. Nonetheless, the utility of this technique in recording vocalizations from individual pair mates during play-fights needs to be evaluated, as the presence of the backpacks may inhibit the play or modify the play that is performed. After all, as already noted above, rolling over on to their backs is an important part of playful wrestling, and the presence of a backpack may hamper such behavior. Also, it is possible that the vigorous nature of play may dislodge the device or obstruct the recording abilities of the microphone. Irrespective of these concerns, the value of gaining information on the vocalizations emitted by individuals during social encounters, and do so while avoiding the potential side effects of surgical manipulation, is so great that these techniques should be tested empirically as tools for studying the USVs used during play-fights.

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References

Aldis O (1975) Play fighting. Academic Press, New York, NY

Altmann SA (1962) Social behavior of anthropoid primates: analysis of recent concepts. In: Bliss EL (ed) Roots of behavior. Harper, New York, pp 277–285

Anisimov VN, Herbst JA, Abramchuk AN, Latanov AV, Hahnloser RH, Vyssotski AL (2014) Reconstruction of vocal interactions in a group of small songbirds. Nat Methods 11:1135–1137

Bekoff M (1975) The communication of play intention: are play signals functional? Semiotica 15:231–239

Berridge KC, Robinson TE (2003) Parsing reward. Trends Neurosci 26:507–513

Blanchard RJ, Blanchard DC (1994) Environmental targets and sensorimotor systems in aggression and defense. In: Cooper SJ, Hendrie CA (eds) Ethology and psychopharmacology. Wiley, New York, NY, pp 133–157

Brudzynski SM (2013) Ethotransmission: communication of emotional states through ultrasonic vocalization in rats. Curr Opin Neurobiol 23:310–317

Burgdorf J, Knutson B, Panksepp J (2000) Anticipation of rewarding brain stimulation evokes ultrasonic vocalizations in rats. Behav Neurosci 97:320–327

Burgdorf J, Knutson B, Panksepp J, Ikemoto S (2001) Nucleus accumbens amphetamine microinjections unconditionally elicit 50-kHz ultrasonic vocalizations in rats. Behav Neurosci 115:940–944

Burgdorf J, Wood PL, Kroes RA, Moskal JR, Panksepp J (2007) Neurobiology of 50-kHz ultrasonic vocalizations in rats: electrode mapping, lesion, and pharmacology studies. Behav Brain Res 182:274–283

Burgdorf J, Kroes RA, Moskal JR, Pfaus JG, Brudzynski SM, Panksepp J (2008) Ultrasonic vocalizations of rats (*Rattus norvegicus*) during mating, play, and aggression: behavioral concomitants, relationship to reward, and self-administration of playback. J Comp Psychol 122:357–367

Fagen R (1981) Animal play behavior. Oxford University Press, New York

Graham KL (2011) Coevolutionary relationship between striatum size and social play in nonhuman primates. Am J Primat 73:314–322

Himmler BT, Pellis VC, Pellis SM (2013) Peering into the dynamics of social interactions: measuring play fighting in rats. J Vis Exp 71:e4288

Himmler BT, Kisko TM, Euston DR, Kolb B, Pellis SM (2014) Are 50-kHz calls used as play signals in the playful interactions of rats? I. Evidence from the timing and context of their use. Behav Process 106:60–66

Hole GJ, Einon DF (1984) Play in rodents. In: Smith PK (ed) Play in animals and children. Blackwell, Oxford, pp 95–117

Kipper S, Todt D (2002) The use of vocal signals in the social play of Barbary macaques. Primates 43:3–17

Kisko TM, Euston DR, Pellis SM (2015a) Are 50-kHz calls used as play signals in the playful interactions of rats? III. The effects of devocalization on play with unfamiliar partners as juveniles and as adults. Behav Process 113:113–121

Kisko TM, Himmler BT, Himmler SM, Euston DR, Pellis SM (2015b) Are 50-kHz calls used as play signals in the playful interactions of rats? II. Evidence from the effects of devocalization. Behav Process 111:25–33

Knutson B, Burgdorf J, Panksepp J (1998) Anticipation of play elicits high-frequency ultrasonic vocalizations in young rats. J Comp Psychol 112:65–73

Lehman MN, Adams DB (1976) A statistical and motivational analysis of the social behaviors of the male laboratory rat. Behaviour, LXI:3–4

Lore R, Flannelly K, Farina P (1976) Ultrasounds produced by rats accompany decreases in intraspecific fighting. Aggress Behav 2:175–181

Lukas M, Wöhr M (2015) Endogenous vasopressin, innate anxiety, and the emission of pro-social 50-kHz ultrasonic vocalizations during social play behavior in juvenile rats. Psychoneuroendocrinology 56:35–44

Moore SJ, Turnpenny P, Quinn A, Glover S, Lloyd DJ, Montgomery T, Dean JCS (2000) A clinical study of 57 children with fetal anticonvulsant syndromes. J Med Genet 37:489–497

Neunuebel JP, Taylor AL, Arthur BJ, Egnor SR (2015) Female mice ultrasonically interact with males during courtship displays. eLife 4:e06203

Palagi E (2011) Playing at every age: modalities and potential functions in non-human primates. In: Pellegrini AD (ed) Oxford handbook of the development of play. Oxford University Press, Oxford, pp 70–82

Palagi E, Burghardt GM, Smuts B, Cordoni G, Dall'Olio S, Fouts HN, Řeháková-Petrů M, Siviy SM, Pellis SM (2015). Rough-and-tumble play as a window on animal communication. Biol Rev, in press

Panksepp J (1981) The ontogeny of play in rats. Dev Psychobiol 14:327–332

Panksepp J, Burgdorf J (2000) 50-kHz chirping (laughter?) in response to conditioned and unconditioned tickle-induced reward in rats: effects of social housing and genetic variables. Behav Brain Res 115:25–38

Pellis SM (1988) Agonistic versus amicable targets of attack and defense: consequences for the origin, function and descriptive classification of play-fighting. Aggress Behav 14:85–104

Pellis SM, McKenna MM (1995) What do rats find rewarding in play fighting? An analysis using drug-induced non-playful partners. Behav Brain Res 68:65–73

Pellis SM, Pellis VC (1983) Locomotor-rotational movements in the ontogeny and play of the laboratory rat (*Rattus norvegicus*). Dev Psychobiol 16:269–286

Pellis SM, Pellis VC (1987) Play-fighting differs from serious fighting in both target of attack and tactics of fighting in the laboratory rat (*Rattus norvegicus*). Aggress Behav 13:227–242

Pellis SM, Pellis VC (1988) Play-fighting in the Syrian golden hamster (*Mesocricetus auratus* waterhouse), and its relationship to serious fighting during post-weaning development. Dev Psychobiol 21:323–337

Pellis SM, Pellis VC (1990) Differential rates of attack, defense and counterattack during the developmental decrease in play fighting by male and female rats. Dev Psychobiol 23:215–231

Pellis SM, Pellis VC (1998) The play fighting of rats in comparative perspective: a schema for neurobehavioral analyses. Neurosci Biobehav Rev 23:87–101

Pellis SM, Pellis VC (2009) The playful brain: venturing to the limits of neuroscience. Oneworld Press, Oxford

Pellis SM, Pellis VC, Himmler BT (2014) How play makes for a more adaptable brain: a comparative and neural perspective. Am J Play 7:73–98

Pellis SM, Pellis VC, Foroud A (2005) Play fighting: aggression, affiliation and the development of nuanced social skills. In: Tremblay R, Hartup WW, Archer J (eds) Developmental origins of aggression. Guilford Press, New York, NY, pp 47–62

Pellis SM, Pellis VC, Reinhart CJ (2010) The evolution of social play. In: Worthman C, Plotsky P, Schechter D, Cummings C (eds) Formative experiences: the interaction of caregiving, culture, and developmental psychobiology. Cambridge University Press, Cambridge, pp 404–431

Pellis SM, Field EF, Smith LK, Pellis VC (1997) Multiple differences in the play fighting of male and female rats. Implications for the causes and functions of play. Neurosci Biobehav Rev 21:105–120

Raza S, Himmler BT, Himmler SM, Harker A, Kolb B, Pellis SM, Gibb R (2015) Effects of prenatal exposure to valproic acid on the development of juvenile-typical social play in rats. Behav Pharmacol 26:707–719

Sadananda M, Wöhr M, Schwarting RKW (2008) Playback of 22-kHz and 50-kHz ultrasonic vocalizations induces differential c-fos expression in rat brain. Neurosci Lett 435:17–23

Sales GD (1972a) Ultrasound and mating behaviour in rodents with some observations on other behavioural situations. J Zool 168:149–164

Sales GD (1972b) Ultrasound and aggressive behaviour in rats and other small mammals. Anim Behav 20:88–100

Schneider T, Przewłocki R (2005) Behavioral alterations in rats prenatally exposed to valproic acid: animal model of autism. Neuropsychopharmacology 30:80–89

Seffer D, Rippberger H, Schwarting RKW, Wöhr M (2015) Pro-social 50-kHz ultrasonic communication in rats: post-weaning but not post-adolescent social isolation leads to social impairments—phenotypic rescue by re-socialization. Front Behav Neurosci 9:102

Sewell GD (1967) Ultrasound in adult rodents. Nature 215:512

Siviy SM, Panksepp J (1987) Sensory modulation of juvenile play in rats. Dev Psychobiol 20:39–55

Siviy SM, Panksepp J (2011) In search of the neurobiological substrates for social playfulness in mammalian brains. Neurosci Biobehav Rev 35:1821–1830

Siviy SM, Harrison KA, McGregor LS (2006) Fear, risk assessment, and playfulness in the juvenile rat. Behav Neurosci 120:49–59

Suomi SJ (2005) Genetic and environmental factors influencing the expression of impulsive aggression and serotonergic functioning in rhesus monkeys. In: Tremblay RE, Hartup WW, Archer J (eds) Developmental origins of aggression. Guilford Press, New York, pp 63–82

Takahashi LK, Thomas DA, Barfield RJ (1983) Analysis of ultrasonic vocalizations emitted by residents during aggressive encounters among rats (*Rattus norvegicus*). J Comp Psychol 97:207–212

Takeuchi H, Kawashima S (1986) Ultrasonic vocalizations and aggressive behavior in male rats. Physiol Behav 38:545–550

Thomas DA, Takahashi LK, Barfield RJ (1983) Analysis of ultrasonic vocalizations emitted by intruders during aggressive encounters among rats (*rattus norvegicus*). J Comp Psychol 97:201–206

Tornatzky W, Miczek KA (1994) Behavioural and autonomic responses to intermittent social stress: differential protection by clonidine and metoprolol. Psychopharmacol 116:346–356

Tornatzky W, Miczek KA (1995) Alcohol, anxiolytics and social stress in rats. Psychopharmacol 121:135–144

Trezza V, Baarendse PJ, Vanderschuren LJ (2010) The pleasures of play: pharmacological insights into social reward mechanisms. Trends Pharmacol Sci 31:463–469

Vanderschuren LJ, Trezza V (2014) What the laboratory rat has taught us about social play behavior: role in behavioral development and neural mechanisms. Curr Top Behav Neurosci 16:189–212

van Hooff J (1967) The facial displays of the catarrhine monkeys and apes. In: Morris D (ed) Primate Ethology. Weidenfeld & Nicolson, London, pp 9–88

van Oortmerssen GA (1971) Biological significance, genetics, and evolutionary origin of variability in behavior within and between inbred strains of mice (*Mus musculus*). Behav 38:1–91

Vivian JA, Miczek KA (1993) Diazepam and gepirone selectively attenuate either 20–32 or 32–64 kHz ultrasonic vocalizations during aggressive encounters. Psychopharmacol (Berl.) 112:66–73

Webber ES, Harmon KM, Beckwith TJ, Peña S, Burgdorf J, Panksepp J, Cromwell HC (2012) Selective breeding for 50 kHz ultrasonic vocalization emission produces alterations in the ontogeny and regulation of rough-and-tumble play. Behav Brain Res 229:138–144

Willey AR, Spear LP (2012) Development of anticipatory 50 kHz USV production to a social stimuli in adolescent and adult male Sprague-Dawley rats. Behav Brain Res 226:613–618

Willuhn I, Tose A, Wanat MJ, Hart AS, Hollon NG, Phillips PE, Schwarting RKW, Wöhr M (2014) Phasic dopamine release in the nucleus accumbens in response to pro-social 50 kHz ultrasonic vocalizations in rats. J Neurosci 34:10616–10623

Wilson SC (1973) The development of social behaviour in the vole (*Microtus agrestis*). Zool J Linn Soc 52:45–62

Wöhr M, Schwarting RKW (2007) Ultrasonic communication in rats: can playback of 50-kHz calls induce approach behavior? PLoS ONE 2:e1365

Wöhr M, Schwarting RKW (2008) Maternal care, isolation-induced infant ultrasonic calling, and their relations to adult anxiety-related behavior in the rat. Behav Neurosci 122:310

Wöhr M, Schwarting RKW (2013) Affective communication in rodents: ultrasonic vocalizations as a tool for research on emotion and motivation. Cell Tissue Res 354:81–97

Wöhr M, Engelhardt AK, Seffer D, Sungur AÖ, Schwarting RKW (2015) Acoustic communication in rats: effects of social experiences on ultrasonic vocalizations as socio-affective signals. Curr Topics Behav Neurosci, in press

Wright JM, Gourdon JM, Clarke PB (2010) Identification of multiple call categories within the rich repertoire of adult rat 50-kHz ultrasonic vocalizations: effects of amphetamine and social context. Psychopharmacol 211:1–13

Yanagi A, Berman CM (2014) Body signals during social play in free-ranging Rhesus macaques (*Macaca mulatta*): a systematic analysis. Am J Primatol 76:168–179

Review II

Title: Playback of ultrasonic vocalizations to juvenile and adult rats: Behavioral and neuronal effects

Authors: Rainer K.W. Schwarting, Theresa M. Kisko, Markus Wöhr

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CHAPTER

34

C0170 # Playback of Ultrasonic Vocalizations to Juvenile and Adult Rats: Behavioral and Neuronal Effects

Rainer K.W. Schwarting, Theresa M. Kisko, Markus Wöhr

Behavioral Neuroscience, Experimental and Biological Psychology, Philipps-University, Marburg, Germany

s0010 I INTRODUCTION

p0010 Although their existence has been known for several decades, ultrasonic vocalizations (USVs) of juvenile or adult rats are currently receiving substantial and increasing attention. They are studied with respect to their roles as social signals, their emotional background, and their underlying brain mechanisms. In this chapter, we present studies of USVs by means of playback techniques, which provide a major approach to analyze their social communicative functions. These techniques were originally introduced to analyze the effects of pup USVs on maternal behavior (e.g., Allin & Banks, 1972; Wöhr & Schwarting, 2008a), but have also received considerable attention in studies of juvenile or adult rats. There, two major call classes can be identified, namely 22-kHz and 50-kHz USVs, related to aversive and appetitive functions, respectively. Playback studies, in which these call types were analyzed, will be reviewed with respect to behavioral and neuronal effects. A similar review has been published before (Wöhr & Schwarting, 2010), and the present one provides a substantial update (see also Q2 Wöhr, Seffer, and Schwarting, 2016).

s0015 II PLAYBACK OF 22-kHz VOCALIZATIONS—EFFECTS ON BEHAVIOR

p0015 A classical hypothesis states that 22-kHz vocalizations serve as alarm calls to warn conspecifics about threats (Blanchard, Blanchard, Agullana, & Weiss, 1991). These authors have shown that the production of 22-kHz calls in response to a predator (a cat) was dependent on the presence of an audience consisting of conspecific rats. This was demonstrated with rats living in a colony in a visible burrow system. The results indicated that the production of vocal signals is not only sensitive to specific eliciting stimuli, but also to the caller's social context, that is, the presence of an identified group of listeners. The calls led to lasting effects in the recipients, even in those who had not experienced the predator themselves (Blanchard, Blanchard, Rodgers, & Weiss, 1990). The findings led to the assumption that the recipients' defensive responses were due to the 22-kHz USVs and not to some other stimulus modalities such as sight or smell. Because the effects of nonacoustic signals could not be completely ruled out in these studies, playback techniques were subsequently supplied to specifically address the impact of USVs and comparable artificial signals on rat behavior.

p0020 Such studies showed that the presentation of natural 22-kHz calls or 20-kHz sine-wave tones to naïve rats can lead to behaviors indicating activation of the fight/flight/freeze system, and that such effects may be partly strain-dependent. Thus, Wistar rats (Brudzynski & Chiu, 1995; Burman, Ilyat, Jones, & Mendl, 2007; Commissaris et al., 2000; Neophytou et al., 2000; Sales, 1991; Wöhr & Schwarting, 2007) and Sprague-Dawley rats (Endres, Widmann, & Fendt, 2007) showed reductions in locomotor activity and moderate freezing, indicating a passive or inhibitory response, whereas Lister hooded rats showed bursts of running and jumping, that is, behavioral excitation, a feature of active defensive behavior (Beckett, Aspley, Graham, & Marsden, 1996; Beckett, Duxon, Aspley, & Marsden, 1997; Commissaris et al., 2000; Commissaris, Beckett, & Marsden, 1998; Neophytou et al., 2000; Nicolas, Klein, & Prinssen, 2007; Voits, Beckett, Marsden, & Fink, 1999).

p0025 When looking in detail, however, the specificity and strength of the behavioral response to playback becomes questionable. Often, effects were only clearly evident with loud and artificial continuous sine-wave tones

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(Beckett et al., 1996, 1997; Commissaris et al., 1998, 2000; Neophytou et al., 2000; Nicolas et al., 2007; Nobre & Brandão, 2004; Voits et al., 1999), whereas behavioral responses were rather weak or absent in the case of natural stimuli (Brudzynski & Chiu, 1995; Burman et al., 2007; Endres et al., 2007; Parsana, Li, & Brown, 2012; Parsana, Moran, & Brown, 2012; Sales, 1991; Wöhr & Schwarting, 2007), indicating that signal features such as loudness have to be controlled carefully.

p0030 In the first study using natural 22-kHz USVs as playback stimuli, Sales (1991) showed that these natural signals led to slight locomotor inhibition, that is, around 20% reduction compared to noise controls. Brudzynski and Chiu (1995) did not find acute effects during playback but decreased locomotor activity thereafter. More recently, Burman et al. (2007) tested a series of 22-kHz calls obtained by manually stroking the back of one of two rats. They found that playback of only one of the two series of USVs was effective in increasing the latency to emerge from a start box into an open arena, compared to background noise. The authors speculated that the two call series might have differed in information content; however, there were no spectrographic comparisons provided to support this assumption. Others (Endres et al., 2007; Wöhr & Schwarting, 2007) observed only nonsignificant changes in response to the playback of natural 22-kHz calls, or no behavioral responses (Bang, Allen, Jones, Boguszewski, & Brown, 2008; Lindquist, Jarrard, & Brown, 2004; Sadananda, Wöhr, & Schwarting, 2008, Parsana, Li, & Brown, 2012; Parsana, Moran, & Brown, 2012).

p0035 Further comparison of responses to natural 22-kHz calls with other ultrasonic stimuli revealed no clear differential response to 22-kHz USVs (Bang et al., 2008; Endres et al., 2007; Sales, 1991). Thus, the reduced locomotor activity during the playback of 22-kHz USVs found by Sales (1991) was similar to that in response to an artificial 38-kHz stimulus. Endres et al. (2007) compared the response to the playback of 22-kHz calls to 50-kHz calls, 22-kHz sine-wave tones, 22-kHz calls shifted to about 45 kHz, and white noise in the range from 17 to 27 kHz. A moderate increase in freezing during and after stimulus presentation was found only when pooling all stimulus types with acoustic features close to 22-kHz USVs, but not selectively in the 22-kHz group itself.

p0040 Furthermore, playback studies with artificial stimuli yielded stronger behavioral responses to 7-kHz or 12-kHz sine-wave tones than to 20-kHz sine-wave tones (Commissaris et al., 2000), indicating that the behavioral effects in these studies were not related to the communicative value of 22-kHz USVs. These and other effects were likely caused by rather high sound pressure levels because some studies used artificial stimuli with more than 100 dB SPL (Commissaris et al., 2000; Voits et al., 1999). This is clearly higher than the usual sound pressure level of natural 22-kHz calls, which is approximately 60–80 dB SPL when measured from a distance of 20–30 cm (Wöhr, Borta, & Schwarting, 2005; Wöhr & Schwarting, 2008a, 2008b). In fact, a critical role of sound pressure in inducing behavioral changes was shown in Lister hooded rats, where the maximum velocity of the stimulus-induced locomotor activity was higher when acoustic stimuli were louder (Commissaris et al., 2000).

p0045 The 22-kHz calls were further used as conditioned stimuli (CS) for fear conditioning (Bang et al., 2008; Endres et al., 2007), where a formerly neutral stimulus gains the efficacy to elicit fear-related conditioned responses (CRs) such as freezing after being paired with an aversive unconditioned stimulus (US). Thus, one can assume that 22-kHz USVs might have no or weak aversive signal features on their own but may easily gain them once they are associated with a substantial aversive experience such as foot shocks, which are well known to elicit 22-kHz calls (Wöhr et al., 2005). Endres et al. (2007) addressed this issue, namely that responding to 22-kHz calls in terms of alarm calls can be learned and that this learning is facilitated by a preparedness to acquire defensive behavioral patterns in response to such stimuli. Rats quickly learned to associate an aversive event with 22-kHz calls, retained this information longer in memory, and were more reluctant to extinguish it than in the case of association of aversive events with other types of ultrasonic stimuli such as artificial 22-kHz sine-wave tones.

p0050 Evidence supporting a predisposition to associate 22-kHz calls with aversive events was also obtained using a differential fear-conditioning paradigm (Bang et al., 2008). Here, 22-kHz calls and other ultrasonic stimuli were used either as CS+, which always coterminated with the US; foot-shock application; or as CS-, presented in an unpaired way. As in the study by Endres et al. (2007), the 22-kHz calls did not differ from the other ultrasonic stimuli in unconditionally eliciting freezing behavior, but after pairing 22-kHz calls and foot shocks, 22-kHz calls alone induced freezing behavior. This suggests that freezing in response to 22-kHz USVs is not innate but may emerge as a consequence of associative learning that is facilitated by a predisposition. The existence of such a predisposition or preparedness to associate certain stimuli with specific consequences was demonstrated before, especially in the case of conditioned taste aversion (Garcia & Koelling, 1966; for review see Seligman, 1970).

p0055 In a similar vein, Parsana, Moran, and Brown (2012) suggested that although the capacity to emit 22-kHz USVs may be innate, the reactivity to such signals may require some form of learning. In laboratory-housed rats, such learning may be rare, which could explain why many studies did not detect substantial behavioral responses in rats receiving playback of 22-kHz calls. Learning to freeze in response to such calls, however, might be acquired in a nonsocial way, namely by

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autoconditioning, which was postulated to occur when the perception of one`s own 22-kHz USVs is associated with a concomitant state of distress or anxiety, leading to freezing or behavioral inhibition.

p0060 The authors tested this hypothesis by using an unsignaled foot-shock procedure, which led to intense 22-kHz vocalization in most animals. On a subsequent day, several of these rats received a playback of a series of 22-kHz calls (previously recorded from another shocked rat) in a test chamber distinct to that used for the shock experience. These rats clearly showed increased freezing to 22-kHz call playback, unlike rats that had not undergone the aversive experience before or when receiving playback of 50-kHz calls. Also, the degree of freezing was descriptively stronger than that in animals receiving artificial 22-kHz tones, which were presented in a continuous manner while natural 22-kHz calls were emitted in bout-like patterns. The authors argued that the effect of 22-kHz USVs on freezing is probably not due to sensitization because freezing to 50-kHz calls should also have occurred in the case of sensitization. Irrespective of these theoretical aspects, the findings (Bang et al., 2008; Endres et al., 2007; Parsana, Moran, & Brown, 2012) suggest that a lack of associative experiences may explain why many playback studies did not find substantial freezing to a presentation of 22-kHz calls or comparable artificial tones. Such lack of experience may be especially relevant in studies where rats were deprived from aversive social encounters due to single housing, as in the case of Parsana, Moran, and Brown (2012).

p0065 Besides prior aversive experiences (see also Kim, Kim, Covey, & Kim, 2010), the presence of conspecific rats during actual testing may play a role for the effectiveness of 22-kHz calls. This idea emerged from the finding that the presence of conspecifics can potentiate 22-kHz call emission (Blanchard et al., 1991). However, such an audience effect was not found in a conventional fear-conditioning paradigm known to induce 22-kHz calling (Borta, Wöhr, & Schwarting, 2006; Wöhr et al., 2005; Wöhr & Schwarting, 2008a), where the experimental rats were tested either alone with an anesthetized conspecific or with an active conspecific in an adjacent chamber. There, the close presence of another rat, if any, had a mild attenuating effect on the call rate of the fear-conditioned rat (Wöhr & Schwarting, 2008b).

p0070 In summary, playback studies with 22-kHz USVs or comparable artificial stimuli have not provided a consistent pattern of behavioral effects on the recipients. These outcomes are due to a number of factors such as the choice of the rat strain. The role of specific stimulus features is less clear because behavioral effects seem not to be selective to 22-kHz signals but perhaps to a broader frequency range, making the question of choosing appropriate control stimuli a challenging and yet unresolved issue. One stimulus feature, namely sound pressure level, is clearly critical because defensive responses are more likely in the case of louder stimuli; yet, care has to be taken to rule out a simple startle response. Also, the testing environment may play a role but this issue has not received substantial attention. In contrast, it has become clear that the rats' behavioral responses to 22-kHz calls depend on an interaction between innate mechanisms and experience. Rats may be predisposed to respond to 22-kHz USV in a defensive manner, but the responses seem to depend on learning and social factors. Therefore, specific experiential background (such as housing, social status, aversive experiences) of the test subjects should routinely be taken into account in future studies.

III PLAYBACK OF 22-kHz VOCALIZATIONS—EFFECTS ON NEURONAL VARIABLES s0020

Several studies using immunohistochemical and electrophysiological techniques have shown that playback of 22-kHz calls or artificial 20-kHz sine-wave tones can lead to specific changes not only in the structures of the auditory system but also in brain areas implicated in the regulation of anxiety and fear. p0075

A Effects of Artificial Ultrasonic Stimuli s0025

Regarding artificial ultrasonic stimuli, Beckett et al. (1997) demonstrated that increased locomotor activity in Lister hooded rats in response to playback of 20-kHz sine-wave tones was paralleled by enhanced neuronal activity in the periaqueductal gray (PAG), amygdala, hypothalamus, and thalamus, measured by means of the immediate early gene c-fos, a rapid cellular marker for neuronal activity. Within these brain sites, activations were predominantly occurring in certain subareas, namely the dorsal PAG; the medial, basolateral, central, and lateral nuclei of the amygdala; and the dorsomedial nucleus of the hypothalamus. Also, the stria terminalis and the paraventricular nucleus of the thalamus–but not the entorhinal cortex–were activated. p0080

Neophytou et al. (2000) examined whether strain differences in behavioral responses to artificial 20-kHz sine-wave tones are paralleled by differences in brain activity. In general, increased activity was observed in the basolateral amygdala, stria terminalis, and entorhinal cortex, but Wistar rats showed less pronounced neuronal activation to playback of 20-kHz sine-wave tones than Lister hooded rats. Most importantly, the pattern of activation in the PAG differed from that of Lister hooded rats. In Lister hooded rats, where playback of 20-kHz tones increased locomotor activity, enhanced neuronal activity was preferentially detected in the dorsal region p0085

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of the rostral and caudal PAG. However, the playback in Wistar rats, which decreased locomotor activity and led to freezing responses, induced activation in the ventral part of the caudal PAG. These regional differences may be functionally important because the PAG presumably represents the final common pathway in the behavioral expression of aversive states (Vianna & Brandão, 2003). It is organized in functional circuits, and electrical or chemical stimulation of the dorsal part of the PAG elicited fleeing while stimulation of the ventral parts of the PAG produced freezing (Depaulis, Keay, & Bandler, 1994; Morgan, Whitney, & Gold, 1998). Furthermore, inactivation of dorsal parts of the PAG increased fear-induced freezing while inactivation of ventral parts disrupted this behavior (DeOca, DeCola, Maren, & Fanselow, 1998).

p0090 Overall, the results of these studies, which did not address structures of the auditory system, showed that artificial 22-kHz call-like stimuli can activate brain regions implicated in the regulation of fear and anxiety.

s0030 B Effects of Natural 22-kHz Calls

p0095 Subsequent studies investigated the effects of natural 22-kHz calls on brain activity, including auditory structures. Using c-Fos immunostaining, Sadananda et al. (2008) showed that playback of 22-kHz USVs leads to sparse activation in the inferior colliculus, especially its central part, and denser labeling in the primary and secondary auditory cortex. Cortical activation showed some tonotopic features and was more pronounced in the left than the right hemisphere, resembling hemispheric lateralization of auditory processing in mice (Ehret, 1987).

p0100 More recently, Ouda, Jilek, and Syka (2016) studied c-Fos expression of female adult Long-Evans rats exposed to the playback of natural 22-kHz USVs, artificial 22-kHz-like sounds, or to live calls emitted by a rat receiving foot shocks in an adjacent cage. In the case of playback, a series of calls was presented in loops for a total of 45 min and frequencies below 8 kHz were filtered out. Foot shock-induced live calls remained unfiltered and had to be induced by a repeated shock paradigm, which led to a more irregular temporal pattern of 22-kHz calls. In contrast, the authors tried to control for comparable sound intensity among these stimulus types. In response to 22-kHz stimuli, activation in the inferior colliculus was detectable throughout the structure. The activation was most pronounced in a band within the central nucleus, probably corresponding to the tonotopic representation of these ultrasonic frequencies. A similar pattern was found in the auditory cortex (especially layers II, III, and VI), except that the degree of activation was less expressed in response to artificial 22-kHz signals. There was no evidence for specific activation in the relevant tonotopic subareas. In contrast to the inferior colliculus and auditory cortex, there was no evidence for activation in the medial geniculate body (see also Chapters 7 and 8 in this volume). The results are generally in line with those of Sadananda et al. (2008) with respect to the playback of natural 22-kHz calls. Unlike, Sadananda et al. (2008), however, no evidence for lateralization was obtained in the auditory cortex. In addition to these data in USV recipients, Ouda et al. (2016) also analyzed c-Fos in the auditory cortex of rats that emitted and heard 22-kHz USVs induced by electric foot shocks. Activations were found in both conditions, but the cortical increase was higher in rats only hearing 22-kHz calls. The lower activation in the auditory cortex of the shocked group may be due to a mechanism of sensory attenuation to self-produced acoustic calls, which has repeatedly been observed in several species (for further discussion, see Ouda et al., 2016).

p0105 Regarding artificial signals, the work of Ouda et al. (2016) supported the results of older studies (Beckett et al., 1997; Neophytou et al., 2000) in showing that these signals can activate relevant auditory and limbic areas. They have also shown that artificial signals were less effective (auditory cortex) or even ineffective (hippocampus, amygdala) to enhance c-Fos activation. It remains to be specified which physical features (e.g., temporal patterning, formants, etc.) of the natural calls made them more effective than the artificial ones.

p0110 Regarding areas implicated in fear and anxiety, evidence with playback of natural 22-kHz USVs was obtained mainly for the PAG, amygdala, and perirhinal cortex (Ouda et al., 2016; Parsana, Li, & Brown, 2012; Sadananda et al., 2008), and some of the results were similar to those obtained with artificial signals (Beckett et al., 1997; Neophytou et al., 2000).

p0115 ***PAG:*** Sadananda et al. (2008) found an increase in the number of activated cells by 22-kHz USV playback, particularly in the rostral part of the PAG. Ouda et al. (2016), who compared natural or artificial 22-kHz calls presented by means of playback, or live calls emitted by a rat receiving foot shocks, found that all stimulus types led to activation in the PAG, with the smallest effect caused by artificial 22-kHz signals.

p0120 The authors also analyzed rats that emitted 22-kHz calls in response to electric foot shocks. Increases in c-Fos-activated cell numbers were found in the PAG; the increases were higher as compared to those in rats receiving only 22-kHz USV playback. This outcome may reflect the substantial involvement of the PAG not only in USV perception and production but also aversion in general, which had a stronger impact on the emission of USVs associated with foot shock than USVs without it.

p0125 ***Amygdala:*** Sadananda et al. (2008) found c-Fos activation especially in the basolateral and lateral part with playback of 22-kHz USVs. Ouda et al. (2016) found that only the natural calls but not artificial 22-kHz signals

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were effective in the activation of the basolateral amygdala, both when presented via playback or when experienced as live calls.

p0130 These c-Fos findings were extended by electrophysiological results obtained by Parsana, Li, and Brown (2012), who collected neuronal responses to natural 22-kHz USVs or continuous 22-kHz tones (matched in terms of frequency but not bout structure). Extracellular recordings were obtained from the lateral and basolateral amygdala. Almost 40% of all units recorded responded to the calls and an even larger percentage (57%) responded to the artificial tones. Many units responded with short latencies and more tonic than phasic activity. Responses to 22-kHz signals were mostly increases in firing rate. This contrasts with results obtained in the same animals in response to 50-kHz signals, which often led to inhibition rather than excitation (see also below).

p0135 The latter results prompted the authors to suggest that some amygdala neurons may differently code the affective valence of aversive versus appetitive ultrasonic signals. Similar conclusions have been drawn in humans when comparing their responses to face stimuli with positive or negative valence (Morris et al., 1996). The USV effect was apparently not determined by the spectral and temporal features of the natural calls because simpler artificial tones were effective in a similar way. Also, the neuronal effects were not dependent on or related to behavioral ones, because these animals, which had no explicit experience with 22-kHz calls before, showed no evidence of freezing or immobility. The authors assumed that the amygdala may be predisposed to respond to certain stimuli with negative valence, a hypothesis that has repeatedly been raised in primates, including humans.

p0140 In general, the effectiveness of 22-kHz calls in increasing amygdala activity adds these signals to the group of other motivationally relevant and negatively valenced stimuli capable of increasing amygdala activity, such as foot shock, restraint, and others (Duncan, Knapp, & Breese, 1996; Kovács, 1998). Based on such findings, it was postulated that the amygdala is a key structure in emotional information processing and fear has been most closely associated with it (e.g. Fendt & Fanselow, 1999; LeDoux, 2000; Maren & Quirk, 2004). The fact that 22-kHz calls yielded an increase in the basolateral–but not in the central amygdala–points to the functional importance of intra-amygdaloid circuits (Pitkänen, Savander, & LeDoux, 1997). The basolateral part is generally considered as the sensory gateway into the amygdala, which receives input from all sensory systems, including auditory. The central amygdala orchestrates appropriate responses to cope with the biologically significant event. In the case of threat, for instance, the output connections of the central amygdala to the brainstem, particularly the PAG, induce freezing. The lack of neuronal activation in the central amygdala in response to 22-kHz USVs found by Sadananda et al. (2008) is therefore in accordance with the observation that 22-kHz calls induced only subtle amounts of freezing or no freezing at all (see above). However, the clear increase in c-Fos expression in the basolateral part of the amygdala may indicate initiation of synaptic changes in this area, which might reflect a learning process or prerequisites to it (Sadananda et al., 2008). This assumption is also supported by results showing that infusion of the GABA agonist muscimol into the basolateral amygdala prior to fear conditioning impaired the acquisition of fear/anxiety to 22-kHz calls (Allen et al., 2008).

p0145 ***Perirhinal cortex:*** Neuronal activation induced by the playback of 22-kHz USVs was also observed in the perirhinal cortex both, by means of c-Fos immunohistochemistry (Sadananda et al., 2008) and electrophysiological methods (Allen, Furtak, & Brown, 2007; Furtak, Allen, & Brown, 2007). This brain area is reciprocally connected with the basolateral amygdala (Pitkänen et al., 1997), and similarly receives rich sensory uni- and multimodal information. Playback studies were pioneered by lesion work showing that perirhinal lesions performed prior to training impaired delayed fear conditioning to 22-kHz calls or artificial 22-kHz call-like stimuli serving as a conditioned stimulus (CS). Such lesions were ineffective when the CS was a continuous tone of the same or lower frequency (Lindquist et al., 2004). This observation was replicated by Kholodar-Smith, Allen, and Brown (2008), who further showed that perirhinal lesions impaired delayed fear conditioning to artificial 22-kHz signals lacking frequency modulation while sparing the temporal patterns of natural 22-kHz calls, which might explain why perirhinal lesions did not affect fear conditioning to a continuous 22-kHz sine-wave tone (Lindquist et al., 2004).

p0150 It was suggested, therefore, that the bout structure of 22-kHz calls is at least part of the reason why normal fear conditioning to 22-kHz calls requires cortical processing whereas such processing is not necessary for conditioning to continuous tones (Allen et al., 2007). In contrast to the delayed fear conditioning, the perirhinal cortex is required in trace fear conditioning to discontinuous and continuous tones (Kholodar-Smith, Boguszewski, & Brown, 2008). The difference between both paradigms is that in the delayed fear conditioning, the unconditional stimulus (US) is presented at the end of the CS whereas in the trace fear conditioning the CS is followed by a trace interval, which is terminated by the US. Probably the role of the perirhinal cortex in trace fear conditioning is distinct from its more perceptual functions in the delayed fear conditioning.

p0155 Allen et al. (2007) investigated whether neurons in the perirhinal cortex are tuned to 22-kHz calls as an important ethological stimulus. They recorded single-unit responses to the presentation of natural 22-kHz USVs

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or control stimuli, namely frequency and temporally matched discontinuous tones, or continuous tones with the same or lower frequencies in freely moving rats. Overall, about 40% of the units responded to one or more of the auditory stimulus types, out of which 69% responded to 22-kHz USVs. Unlike the continuous tones, however, the 22-kHz calls sometimes elicited call-related firing patterns. They consisted of a transient increase in the firing rate triggered by the onset, or less often the offset, of each of the successive calls in a bout of calls; similar firing patterns were elicited by the discontinuous tones. Therefore, Allen et al. (2007) concluded that the naturally occurring frequency modulation associated with individual calls does not affect firing pattern or the overall level of responsiveness. Later, Parsana, Li, and Brown (2012) compared these results to the ones obtained in the amygdala and concluded that the perirhinal cortex seems to be even more responsive to 22-kHz signals, particularly in terms of call-related firing patterns.

p0160 Furtak et al. (2007) used a classical fear-conditioning paradigm in which 22-kHz USVs or continuous 22-kHz sine-wave tones served as the CS. Perirhinal firing changes were observed in about 70% of the recorded units in response to 22-kHz calls or tones after pairing them with foot shocks (US). Conditioning caused widespread changes in neuronal firing regardless of whether 22-kHz USVs or a 22-kHz sine-wave tone served as a cue and about 30% of initially unresponsive units became CS-responsive after conditioning.

p0165 In addition, two differences between responses elicited by 22-kHz USVs versus a 22-kHz sine-wave tone became evident. First, about 10% of the units recorded from the group that was conditioned to 22-kHz calls displayed a precisely timed increase in firing rate during the interval, in which the US occurred during conditioning. This response pattern was not seen in rats conditioned to a 22-kHz sine-wave tone. Second, it was found that conditioning decreased response latencies in a stimulus-specific way. Before conditioning, the neurons started firing to both CS after about 55 ms. After conditioning, however, these latencies became shorter in response to 22-kHz sine-wave tones but not the 22-kHz USVs. The authors suggested that before conditioning, firing to both stimulus types was mediated by cortical rather than subcortical pathways to the perirhinal cortex, but that subcortical pathways gained control of firing through conditioning to 22-kHz sine-wave tones but not 22-kHz USVs (Furtak et al., 2007).

p0170 In summary, the current studies on neuronal responses to playback of 22-kHz signals indicate that the presentation of 22-kHz calls (and partly artificial 22-kHz signals) not only activates relevant auditory brain areas but also brain regions implicated in the regulation of fear/anxiety and defense mechanisms in the receiver. These areas include the PAG, amygdala, and perirhinal cortex, where stimulus-specific activations were found in relevant subareas. Also, the current evidence shows that parts of these regions are required for fear conditioning to 22-kHz calls, and among them, the perirhinal cortex might be part of the "neural template" (Endres et al., 2007) responsible for the biological preparedness to associate 22-kHz calls with aversive events.

IV PLAYBACK OF 50-kHz VOCALIZATIONS—EFFECTS ON BEHAVIOR s0035

p0175 The hypothesis that 50-kHz calls serve as contact calls was originally based on behavioral observations in the sexual context, where 50-kHz USVs are emitted by males and females while approaching and investigating the partner (Sales, 1972; Thomas & Barfield, 1985).

p0180 A functional effect of male 50-kHz calls upon female proceptivity was indicated by devocalization and playback studies. When adult males were devocalized by resection of the laryngeal nerves and mated with estrous females, a reduced rate of darting and ear wiggling was observed in these females in comparison to other females mated with sham-operated controls (Thomas, Talalas, & Barfield, 1981). Furthermore, although playback of male 50-kHz calls to a cage with solitary estrous females had no obvious effect on their behavior (Geyer & Barfield, 1978), females showed an increased level of proceptive behavior if they were exposed to males immediately after playback. Male 50-kHz USV playback could even restore proceptive behavior in estrous females when mated with devocalized males (Geyer & Barfield, 1978; McIntosh, Barfield, & Geyer, 1978; White & Barfield, 1990). Finally, and most intriguingly, when male 50-kHz calls were presented while females were in contact with a castrated nonmating male, the females showed elevated solicitation behavior and even lordosis responses without being mounted (McIntosh et al., 1978). Playback of male 50-kHz USVs also led to USV emission by female recipients (White, Gonzales, & Barfield, 1993). Their 50-kHz calls also appear to be important for mating because devocalized females received fewer intromissions despite displaying enhanced darting and approaches towards the partner. Moreover, normal mating activity was restored when tape-recorded female USVs were presented to such pairs (White & Barfield, 1987, 1989).

p0185 More recently, it has become apparent that 50-kHz calls also serve communicative purposes in nonsexual contexts. Panksepp, Gordon, and Burgdorf (2002) observed that rats spent more time with conspecifics that vocalized at high rates, rather than with those that displayed less calling behavior. This observation suggested that 50-kHz calls are used as contact calls to (re)establish

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or to maintain contact among conspecifics. Such a view is supported by different lines of research.

p0190 First, Siviy and Panksepp (1987) showed that deafening rats can affect juvenile play, a situation where 50-kHz calls are typically emitted (Knutson, Burgdorf, & Panksepp, 1998). Further support comes from devocalization studies done by Kisko, Himmler, Himmler, Euston, and Pellis (2015), who demonstrated that the rate of play significantly decreases when neither rat can emit 50-kHz USVs. However, when one partner can vocalize, the rate of play returns to levels seen in controls. In addition, as adults play can be used by rats to resolve ambiguous situations and determine dominance (Smith, Fantella, & Pellis, 1999). Evidence suggests that USVs, in this case, may serve as appeasement signals keeping the interaction from becoming aggressive. For example, the rate of an encounter becoming physically aggressive is significantly greater in pairs of unfamiliar rats in a neutral arena if one of them has been devocalized rather than if both rats are able to emit USVs (Kisko, Euston, & Pellis, 2015).

p0195 Second, Brudzynski and Pniak (2002) found that rats emitted 50-kHz calls when exposed to conspecific odor, with the number of emitted calls being directly proportional to the number of rats leaving their odor. This result indicated that the production of 50-kHz calls is driven by potential social contact.

p0200 Third, the emission of 50-kHz USVs was also detected after separation of cage mates from each other during short social isolation in the animal's own or an unfamiliar soiled or fresh housing cage (Schwarting, Jegan, & Wöhr, 2007; Wöhr, Houx, Schwarting, & Spruijt, 2008). Remarkably, the propensity to call differed depending on the timepoint of the last social contact with rats that emitted 50-kHz USVs after separation from their cage mate (Wöhr et al., 2008). Last, it was found that not only the animal isolated in a new housing cage emitted 50-kHz calls, but also that calls were emitted by the cage mate that remained alone in the home cage (Wöhr et al., 2008).

p0205 In order to gauge the communicative value of 50-kHz calls without the presence of another rat, we developed a playback paradigm that enabled us to measure the effects of natural USVs or various control stimuli on rat approach behavior and emission of USVs. For this purpose, we adopted an eight-arm radial maze (Wöhr & Schwarting, 2007; for details see Seffer, Schwarting, & Wöhr, 2014), to which the animal was habituated before presentation of ultrasonic stimuli provided by loudspeakers placed outside the maze radius and in the same horizontal plane as the arm surface. Stimuli were presented repeatedly for 1 or 5 min and responses to playback were evaluated mainly in terms of activity in arms situated proximal versus distal to the active speaker. The effects clearly demonstrated that playback of 50-kHz calls can induce approach behavior and emission of USVs (Brenes et al., 2016; Seffer, Rippberger, Schwarting, & Wöhr, 2015; Willadsen, Seffer, Schwarting, & Wöhr, 2014; Willuhn et al., 2014; Wöhr & Schwarting, 2009, 2012). These results support the hypothesis that 50-kHz USVs are used to (re)establish or to maintain contact among conspecifics (Brudzynski & Pniak, 2002; Panksepp & Burgdorf, 2003; Schwarting et al., 2007; Siviy & Panksepp, 1987; Wöhr et al., 2008). Q4

p0210 In detail, the exposure of adult male rats to 50-kHz USVs induced a three-fold increase in locomotor activity in comparison to that recorded without playback or with presentation of background noise (Wöhr & Schwarting, 2007). Furthermore, activity was directed toward the loudspeaker during playback and adult rats spent twice as much time close to speakers than away from them. This effect was dependent on the animals' developmental stage because the preference was clearly more pronounced in juvenile rats. Also, juvenile rats were more likely than adults to emit USVs themselves (Wöhr & Schwarting, 2009). This juvenile sensitivity is in line with the fact that 50-kHz USVs play an important prosocial role during juvenile rough-and-tumble play (Knutson et al., 1998).

p0215 The relationship between play and 50-kHz calls was supported by pharmacological studies (Wöhr & Schwarting, 2009). Because it is known that endogenous opioids are implicated in the regulation of social behavior, particularly rough-and-tumble play (for reviews see Panksepp, Siviy, & Normansell, 1984, Vanderschuren, RJM, & Van Ree, 1997), we tested whether the administration of opioid ligands can affect approach in response to playback of 50-kHz calls in juvenile and adult rats. The animals were either treated with the opioid antagonist naloxone or the agonist morphine prior to the playback test. The treatments affected the social approach to 50-kHz USV playback at both ages, with the reduction of the approach caused by naloxone but the enhancement with morphine. Furthermore, juvenile rats treated with saline or morphine emitted USVs in response to the 50-kHz USV playback, which was not observed after application of the antagonist. These drug-dependent differences were stimulus-specific, that is, they were not seen during background noise exposure. The findings indicated that the emission of USVs is an important feature of social interaction in rats and at least partially regulated by endogenous opioids.

p0220 Furthermore, we found that social approach and USV emission occurred specifically in response to signals within the 50-kHz USV range of frequencies, because no such responses were observed when rats were exposed to background noise or 22-kHz USVs. The fact that both 50-kHz calls and 50-kHz sine-wave tones led to behavioral changes in the recipients indicated that amplitude and frequency modulation carried little or no communicative information. In contrast, the peak frequency of 50-kHz calls seems to be behaviorally relevant, as shown in a subsequent study (Wöhr & Schwarting, 2012).

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p0225 Apart from rat age and acoustic features, social approach behavior in response to the playback of 50-kHz USVs is apparently modulated by social memory. Such memory processes are indicated by the observation that social approach behavior is most evident during the first exposure to 50-kHz USV playback but not during repeated exposures (Wöhr & Schwarting, 2012). Even with a one-week interval between exposures, no social approach behavior was found during the retest, suggesting that social long-term memory processes are involved. Evidence in favor of this hypothesis was obtained by pharmacological manipulation using scopolamine, a muscarinic acetylcholine antagonist that leads to amnesia (e.g., D'Amato & Moles, 2001). We found that long-term memory effects caused by 50-kHz USV playback were blocked by the administration of scopolamine immediately after the first exposure (Wöhr & Schwarting, 2012), that is, during the presumptive memory consolidation phase. No such effect was seen in saline-treated controls.

p0230 Others (Burman et al., 2007) did not detect a behavioral response in male rats to 50-kHz USV playback recorded from a male rat exploring an empty and clean housing cage. The lack of an effect compared to our results (e.g., Wöhr & Schwarting, 2007) is probably due to the testing environment. Burman et al. (2007) measured whether playback would affect emergence from a small start box into a larger circular arena–a test usually used to measure anxiety (e.g., Paré, Tejani-Butt, & Kluczynski, 2001). Possibly, approach effects of 50-kHz USV playback are less likely or unlikely under such conditions. Also, in Burman et al. (2007) study, playback was delivered by a loudspeaker positioned above the arena whereas we placed our loudspeakers in the same horizontal plane as the animal, which might make an approach response more appropriate and likely.

p0235 Recently, it was postulated that male 50-kHz USVs do not have incentive values for female rats (Snoeren & Agmo, 2014). This conclusion was based on a study in sexually receptive female rats that received playback recorded from male rats during a precopulatory phase. In this study, flat calls, frequency-modulated calls, or a natural series including all these types were presented in a test arena, which the experimental female rat could approach but not enter. In order to test whether these negative findings might be due to methodological aspects, we tested female sexually experienced rats in our routine playback paradigm with the radial maze set-up (Wöhr & Schwarting, 2007). Similar to our studies with male recipients, we found that the females also showed more approach (but not more activity) toward the side of the loudspeaker presenting 50-kHz calls whereas background noise tended to inhibit behavioral activity (Willadsen et al., 2014). Compared to our previous work with males, the females' approaches were even more pronounced.

p0240 Regarding the reasons that may have accounted for the discrepancies between the results of Snoeren and Agmo (2014) and our results, the experience of female test subjects has to be considered. Snoeren and Agmo used ovariectomized and progesterone-treated females that had not delivered pups whereas we tested untreated females that had delivered and raised pups several weeks before the playback test. This maternal factor, however, does not explain why Snoeren and Agmo (2013) did not find an effect in males. Therefore, other factors might have been more important. For example, Snoeren and Agmo used repeated stimulus exposures without subsequent social consequences, and as explained above, the approach effect disappears rapidly with repeated testing (Wöhr & Schwarting, 2012). Evidence in favor of such an explanation also comes from work with female mice that showed approach to male USVs during the first presentation but not during subsequent ones (Hammerschmidt, Radyushkin, Ehrenreich, & Fischer, 2009). Probably, rats (and mice) can learn rather quickly when an incentive stimulus in a certain context is not followed by the signaled social goal.

p0245 In summary, the studies on behavioral responses to 50-kHz calls accumulated so far indicate that 50-kHz USVs can exert several appetitive effects. These can serve to establish or maintain social contact with conspecifics such as in play and sexual interactions, but also in other social encounters of juvenile and adult male and female rats. Regarding the approach-eliciting features of 50-kHz USVs, methodological details seem to play a critical role for animal responses, such as the testing environment. Also, experience with 50-kHz USV playback seems to be important because rats rapidly habituate to the playback and stop responding.

V PLAYBACK OF 50-kHz VOCALIZATIONS—NEURONAL EFFECTS

s0040

p0250 Compared to results with 22-kHz USVs, the neuronal effects of 50-kHz calls have as yet received little attention. Sadananda et al. (2008) showed that playback of a series of 50-kHz USVs led to only a few changes in c-Fos labeling in the brain as compared to the effects of 22-kHz USVs. Interestingly, most of these changes indicated decreased neuronal activation as compared to nonstimulated controls. The reasons for these decreases are unclear. They were observed in the central amygdala, the lateral habenula, and the dorsal raphé nuclei. Similar trends of decrease were also observed in the 22-kHz USV group (nonsignificantly in the lateral habenula); therefore, they were probably not specific to the stimulus value, that is, aversive versus appetitive valence.

p0255 Apart from these decreases, increased numbers of c-Fos-labeled cells after 50-kHz USV playback were

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observed in the frontal cortex and in the nucleus accumbens (Sadananda et al., 2008, see also Pultorak et al., 2016). Cortical neuronal activation was measured in the secondary motor cortex and was specific to the 50-kHz USV playback group. This activation was probably associated with the observation that only this type of ultrasonic stimulation was effective in inducing pronounced behavioral activation, which was mainly directed toward the source of the stimulation.

p0260 Such activation and social approach might also explain why the nucleus accumbens showed some signs of activation. The nucleus accumbens is well known for its critical role in motivated behavior, especially as an "interface between motivation and action" (Mogenson, Jones, & Yim, 1980). It is important for locomotor activity and approach behavior, and both are critically modulated by its dopaminergic input. Therefore, one can assume that dopaminergic activation in the nucleus accumbens was necessary for approach towards appetitive 50-kHz calls. Besides, this brain area is also efficient in eliciting 50-kHz USVs but not 22-kHz calls, for example, by local administration of amphetamine, a catecholaminergic agonist with strong dopaminergic effects (Burgdorf, Knutson, Panksepp, & Ikemoto, 2001; Thompson, Leonard, & Brudzynski, 2006). Possibly, the nucleus accumbens serves to close the functional link between mechanisms of detection and production of 50-kHz calls, which seems to be especially relevant in the case of appetitive social and reciprocal communicatory signals. Such a link might help to explain why juvenile play, which is accompanied and maintained by increased emission of 50-kHz USVs, also leads to enhanced c-Fos labeling in the nucleus accumbens (Gordon, Kollack-Walker, Akil, & Panksepp, 2002).

p0265 The link to dopamine in the nucleus accumbens was addressed specifically in a recent playback study where extracellular dopamine was monitored *in vivo* by means of fast-scan cyclic voltammetry (Willuhn et al., 2014) in awake rats experiencing various kinds of USV playbacks. When presented with 50-kHz USVs (calls as in Wöhr & Schwarting, 2007), there was a rapid increase of extracellular dopamine in the nucleus accumbens, which was paralleled by behavioral activation and approach toward the signal source. Importantly, the dopamine increase was positively correlated with approach but not locomotor activity, indicating that the neurochemical correlate was not simply associated with general behavioral activation. No dopaminergic effects were observed after 22-kHz USV playback, background noise, or time- and amplitude-matched noise, indicating that the dopamine response to 50-kHz calls was stimulus-selective.

p0270 Interestingly, the behavioral response to 50-kHz USV playback again habituated quickly with repeated presentations (see also Wöhr & Schwarting, 2012), and this behavioral habituation was paralleled by a neurochemical one. This outcome supports the suggested link between 50-kHz USVs, approach, and nucleus accumbens dopamine, and indicates that repetitive 50-kHz calls may rapidly lose their incentive properties, at least when not followed by appropriate social consequences.

p0275 Regarding other motivationally and emotionally relevant brain areas, Parsana, Li, and Brown (2012) recently analyzed neuronal firing activity in the lateral amygdala in rats receiving playback of 50-kHz USVs or artificial and amplitude-matched continuous 50-kHz tones. These USV stimuli affected the firing rate in the amygdala, and in many cases the response was a tonic decrease in firing rate, which contrasts with the increases obtained when presenting 22-kHz calls or tones. Moreover, latencies of these changes differed between stimulus classes, that is, they were longer in response to 50-kHz signals. The authors suggested that 50-kHz signals may take a longer route (thalamus-neocortex-amygdala) than 22-kHz calls (thalamus-amygdala), which may partly reflect the preferential processing of the latter.

p0280 Overall, the current literature shows that playback of 50-kHz USVs leads to neuronal changes in the brain, which can be detected at the immunohistochemical, neurochemical, and electrophysiological levels. These changes are brain site-dependent and can be increases or decreases in neuronal firing rates. Furthermore, the effects appear to be stimulus-selective, particularly in the lateral amygdala, frontal cortex, and nucleus accumbens. In the nucleus accumbens, the approach-eliciting properties of 50-kHz USV stimuli seem to be linked to dopamine activity, which indicates that these effects are processed in the same sites as other appetitive motivationally relevant stimuli.

VI APPLICATION OF 50-kHz USV PLAYBACK APPROACHES IN PRECLINICAL STUDIES

s0045

p0285 Recently, publications have started to appear noting that rat responses to USV signals are considered as functional markers with relevance to human diseases and disorders of the brain.

p0290 In rat models of Parkinson's disease, it was shown that such animals not only produce impaired 50-kHz USVs (Ciucci et al., 2009), but that playback of such calls (Pultorak et al., 2016) is also less efficient at inducing approach in recipients. This outcome resembles the clinical situation because Parkinson's patients can show prosodic deficits (e.g., Ho, Iansek, Marigliani, Bradshaw, & Gates, 1998), which can result in impaired oral communication with others. Interestingly, impaired USVs also led to less c-Fos expression in the recipient's nucleus accumbens (Pultorak et al., 2016), supporting previous findings that 50-kHz USV-induced approach is related to neural

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activation in the nucleus accumbens (Sadananda et al., 2008; Willuhn et al., 2014).

p0295 Furthermore, USV playback was applied in a test of cognitive bias (Saito, Yuki, Seki, Kagawa, & Okanoya, 2016), an instrumental paradigm potentially modeling cognitive changes of human depression, otherwise difficult to address in rodents. The authors found that USV playback prior to instrumental testing affected subsequent performance in a stimulus-specific manner. The 50-kHz USV playback led to more "optimistic" responding compared to more "pessimistic" responses after 22-kHz USV. Saito et al. (2016) suggested that USV playback affected the rats' emotional states in terms of "emotional contagion" with 50-kHz signals leading to a positive emotional state and 22-kHz USV to a negative one.

p0300 Finally, the playback of 50-kHz USVs has been used to test for social impairments in rats that underwent social deprivation during the juvenile phase, where 50-kHz calls serve a substantial role for the animals' rough-and-tumble play (see also Chapter 35 in this issue). Such early social deprivation can lead to alterations in adulthood, including cognitive and social impairments such as social withdrawal. Therefore, juvenile social deprivation and its consequences serve as a model for neurodevelopmental disorders such as autism and schizophrenia characterized by social communication deficits and cognitive inflexibility (e.g. Fone & Porkess, 2008). We have recently shown that four weeks of social isolation starting at weaning impaired the approach response to 50-kHz USV playback whereas there were no deprivation effects in response to 22-kHz USV playback (Seffer et al., 2015). The 50-kHz USV effects were specific to the four postweaning weeks since social isolation started thereafter was not effective, underlining the importance of social experience during the juvenile phase. Our findings are in line with numerous studies reporting alterations in social behavior following postweaning isolation. They add the 50-kHz USV playback technique as a new and promising approach to study preclinical models for disorders such as autism or schizophrenia, where socioaffective information processing is impaired.

p0305 In summary, studying the playback of 50-kHz USVs has proven successful in preclinical models relevant for Parkinson's disease, depression, and negative environmental impacts. Obviously, the application of rat 50-kHz USV playback in preclinical models is currently still in its infancy, but will surely receive more attention in future studies where social deficits in the recipient rather than the sender are of interest for a given disorder.

VII CONCLUSIONS

s0050

p0310 The current evidence obtained in rat playback studies with natural or artificial 22- or 50-kHz USVs shows that behavioral responses to such playback can depend on a number of methodological and subject-dependent factors, including signal features (loudness or call patterns), testing environment, rat strain, and prior experiences of the receivers. In the case of 22-kHz calls, several behavioral and neuronal responses to their presentation support the hypothesis that this call type serves as an alarm call. However, such signals seem not to be innately recognized as alarm calls but can obtain their signal value as a consequence of social and nonsocial associative learning. Such learning is apparently facilitated by a biological preparedness to associate 22-kHz USVs with aversive events. Regarding neural mechanisms, several brain structures beyond auditory ones seem to play a role, including the PAG, amygdala, and perirhinal cortex, with the latter possibly providing part of the "neural template" responsible for such biological preparedness. Behavioral and neuronal responses to 50-kHz USVs indicate that such calls can serve as appetitive contact signals for juvenile and adult male and female rats where calls are relevant in several contexts such as play, sexual interaction, and others. Social approach in response to 50-kHz calls is paralleled by changes in several brain areas, especially the nucleus accumbens. There, 50-kHz USVs evoke phasic dopamine release, which might be related to the appetitive value of these auditory signals. Together, it can be concluded that 22-kHz and 50-kHz USVs represent two behaviorally opposite classes of USVs associated with aversive/defensive or affiliative/appetitive functions, respectively. Furthermore, they are related to specific and partly distinct brain regions. In rat models for neurological and neuropsychiatric disorders, evidence is accumulating that the assessment of behavioral responses elicited in recipients through the playback of 22- and 50-kHz USVs might allow the detection of disease-relevant phenotypes, including impairments in socioaffective information processing that can be relevant in preclinical models of Parkinson's disease, depression, autism, schizophrenia, and other disorders where social deficits play a role.

References

Allen, T. A., Furtak, S. C., & Brown, T. H. (2007). Single-unit responses to 22 kHz ultrasonic vocalizations in rat perirhinal cortex. *Behavioural Brain Research*, *182*, 327–336.

Allen, T. A., Narayanan, N. S., Kholodar-Smith, D. B., Zhao, Y., Laubach, M., & Brown, T. H. (2008). Imaging the spread of reversible brain inactivations using fluorescent muscimol. *Journal of Neuroscience Methods*, *171*, 30–38.

Allin, J. T., & Banks, E. M. (1972). Functional aspects of ultrasound production by infant albino rats (*Rattus norvegicus*). *Animal Behavior*, *20*, 175–185.

Bang, S. J., Allen, T. A., Jones, L. K., Boguszewski, P., & Brown, T. H. (2008). Asymmetrical stimulus generalization following differential fear conditioning. *Neurobiology of Learning and Memory*, *90*, 200–216.

HBBN, 978-0-12-809600-0

B978-0-12-809600-0.00034-2

Beckett, S. R. G., Aspley, S., Graham, M., & Marsden, C. A. (1996). Pharmacological manipulation of ultrasound induced defence behaviour in the rat. *Psychopharmacology*, *127*, 384–390.

Beckett, S. R. G., Duxon, M. S., Aspley, S., & Marsden, C. A. (1997). Central c-fos expression following 20kHz/ultrasound induced defence behaviour in the rat. *Brain Research Bulletin*, *42*, 421–426.

Blanchard, R. J., Blanchard, D. C., Agullana, R., & Weiss, S. M. (1991). Twenty-two kHz alarm cries to presentation of a predator, by laboratory rats living in visible burrow systems. *Physiology and Behavior*, *50*, 967–972.

Blanchard, R. J., Blanchard, D. C., Rodgers, J., & Weiss, S. M. (1990). The characterization and modelling of antipredator defensive behavior. *Neuroscience and Biobehavioral Reviews*, *14*, 463–472.

Borta, A., Wöhr, M., & Schwarting, R. K. W. (2006). Rat ultrasonic vocalization in aversively motivated situations and the role of individual differences in anxiety-related behavior. *Behavioural Brain Research*, *166*, 271–280.

Brenes, J. C., Lackinger, M., Höglinger, G. U., Schratt, G., Schwarting, R. K. W., & Wöhr, M. (2016). Differential effects of social and physical environmental enrichment on brain plasticity, cognition, and ultrasonic communication in rats. *Journal of Comparative Neurology*, *524*, 1586–1607.

Brudzynski, S. M., & Chiu, E. M. C. (1995). Behavioural responses of laboratory rats to playback of 22 kHz ultrasonic calls. *Physiology and Behavior*, *57*, 1039–1044.

Brudzynski, S. M., & Pniak, A. (2002). Social contacts and production of 50-kHz short ultrasonic calls in adult rats. *Journal of Comparative Psychology*, *116*, 73–82.

Burgdorf, J., Knutson, B., Panksepp, J., & Ikemoto, S. (2001). Nucleus accumbens amphetamine microinjections unconditionally elicit 50-kHz ultrasonic vocalizations in rats. *Behavioral Neuroscience*, *115*, 940–944.

Q5 Burgdorf, J., Kroes, R. A., Moskal, J. R., Pfaus, J. G., Brudzynski, S. M., & Panksepp, J. (2008). Ultrasonic vocalizations of rats (*Rattus norvegicus*) during mating, play, and aggression: behavioral concomitants, relationship to reward, and self-administration of playback. *Journal of Comparative Psychology*, *122*, 357–367.

Burman, O. H. P., Ilyat, A., Jones, G., & Mendl, M. (2007). Ultrasonic vocalizations as indicators of welfare for laboratory rats (*Rattus norvegicus*). *Applied Animal Behavior Sciences*, *104*, 116–129.

Ciucci, M. R., Ahrens, A. M., Ma, S. T., Kane, J. R., Windham, E. B., Woodlee, M. T., et al. (2009). Reduction of dopamine synaptic activity: degradation of 50-kHz ultrasonic vocalization in rats. *Behavioral Neuroscience*, *123*, 328–336.

Commissaris, R. L., Beckett, S. R. G., & Marsden, C. A. (1998). Strychnine effects on ultrasound-elicited behaviours in Lister hooded rats. *Psychopharmacology*, *136*, 162–171.

Commissaris, R. L., Palmer, A., Neophytou, S. I., Graham, M., Beckett, S. R. G., & Marsden, C. A. (2000). Acoustically elicited behaviours in Lister hooded and Wistar rats. *Physiology and Behavior*, *68*, 521–531.

D'Amato, F. R., & Moles, A. (2001). Ultrasonic vocalizations as an index of social memory in female mice. *Behavioral Neuroscience*, *115*, 834–840.

DeOca, B. M., DeCola, J. P., Maren, S., & Fanselow, M. J. (1998). Distinct regions of the periaqueductal grey are involved in the acquisition and expression of defensive responses. *The Journal of Neuroscience*, *18*, 3426–3443.

Depaulis, A., Keay, K. A., & Bandler, R. (1994). Longitudinal neuronal organization of defensive reactions in the midbrain periaqueductal gray region of the rat. *Experimental. Brain Research*, *90*, 307–318.

Duncan, G. E., Knapp, D. J., & Breese, G. R. (1996). Neuroanatomical characterization of Fos induction in rat behavioral models of anxiety. *Brain Research*, *713*, 79–91.

Ehret, G. (1987). Left hemisphere advantage in the mouse brain for recognizing ultrasonic communication calls. *Nature*, *325*, 249–251.

Endres, T., Widmann, K., & Fendt, M. (2007). Are rats predisposed to learn 22 kHz calls as danger-predicting signals? *Behavioural Brain Research*, *185*, 69–75.

Fendt, M., & Fanselow, M. S. (1999). The neuroanatomical and neurochemical basis of conditioned fear. *Neuroscience and Biobehavioral Reviews*, *23*, 743–760.

Fone, K. C. F., & Porkess, M. V. (2008). Behavioural and neurochemical effects of post-weaning social isolation in rodents - relevance to developmental neuropsychiatric disorders. *Neuroscience and Biobehavioral Reviews*, *32*, 1087–1102.

Furtak, S. C., Allen, T. A., & Brown, T. H. (2007). Single-unit firing in rat perirhinal cortex caused by fear conditioning to arbitrary and ecological stimuli. *The Journal of Neuroscience*, *27*, 12277–12291.

Garcia, J., & Koelling, R. (1966). Relation of cue to consequence in avoidance learning. *Psychonomic Science*, *4*, 123–124.

Geyer, L. A., & Barfield, R. J. (1978). Influence of gonadal hormones and sexual behavior on ultrasonic vocalization in rats: I. Treatment of females. *Journal of Comparative and Physiological Psychology*, *92*, 436–446.

Gordon, N. S., Kollack-Walker, S., Akil, H., & Panksepp, J. (2002). Expression of c-*fos* gene activation during rough and tumble play in juvenile rats. *Brain Research Bulletin*, *57*, 651–659.

Hammerschmidt, K., Radyushkin, K., Ehrenreich, H., & Fischer, J. (2009). Female mice respond to male ultrasonic 'songs' with approach behavior. *Biology Letters*, *5*, 589–592.

Ho, A. K., Iansek, R., Marigliani, C., Bradshaw, J. L., & Gates, S. (1998). Speech impairment in a large sample of patients with Parkinson's disease. *Behavioral Neurology*, *11*, 131–137.

Inagaki, H., Kuwahara, M., Tsubone, H., & Mori, Y. (2013). The effect of Q6 post-weaning individual housing on 50-kHz calls emitted from male rats to sexually receptive female rats. *Physiology and Behaviour*, *110–111*, 30–33.

Kholodar-Smith, D. B., Allen, T. A., & Brown, T. H. (2008). Fear conditioning to discontinuous auditory cues requires reirhinal cortical function. *Behavioral Neuroscience*, *122*, 1178–1185.

Kholodar-Smith, D. B., Boguszewski, P., & Brown, T. H. (2008). Auditory trace fear conditioning requires perirhinal cortex. *Neurobiology of Learning and Memory*, *90*, 537–543.

Kim, E. J., Kim, E. S., Covey, E., & Kim, J. J. (2010). Social transmission of fear in rats: the role of 22-kHz ultrasonic distress vocalization. *PLoS One*, *5*(12), 15077. https://doi.org/10.1371/journal.pone.0015077.

Kisko, T. M., Euston, D. R., & Pellis, S. M. (2015). Are 50-kHz calls used as play signals in the playful interactions of rats? III. The effects of devocalization on play with unfamiliar partners as juveniles and as adults. *Behavioural Processes*, *113*, 113–121.

Kisko, T. M., Himmler, B. T., Himmler, S. M., Euston, D. R., & Pellis, S. M. (2015). Are 50-kHz calls used as play signals in the playful interactions of rats? II. Evidence form the effects of devocalization. *Behavioural Processes*, *111*, 25–33.

Knutson, B., Burgdorf, J., & Panksepp, J. (1998). Anticipation of play elicits high-frequency ultrasonic vocalizations in young rats. *Journal of Comparative Psychology*, *112*, 65–73.

Kovács, K. J. (1998). c-fos as a transcription factor: a stressful (re)view from a functional map. *Neurochemistry International*, *33*, 287–297.

LeDoux, J. E. (2000). Emotion circuits in the brain. *Annual Review of Neuroscience*, *23*, 155–184.

Lindquist, D. H., Jarrard, L. E., & Brown, T. H. (2004). Perirhinal cortex supports delay fear conditioning to rat ultrasonic social signals. *The Journal of Neuroscience*, *24*, 3610–3617.

Maren, S., & Quirk, G. J. (2004). Neuronal signaling of fear memory. *Nature Reviews Neuroscience*, *5*, 844–852.

McIntosh, T. K., Barfield, R. J., & Geyer, L. A. (1978). Ultrasonic vocalisations facilitate sexual behaviour of female rats. *Nature*, *272*, 163–164.

Mogenson, G. J., Jones, D. L., & Yim, C. Y. (1980). From motivation to action: functional interface between the limbic system and the motor system. *Progress in Neurobiology*, *14*, 69–97.

HBBN, 978-0-12-809600-0

B978-0-12-809600-0.00034-2

Morgan, M. M., Whitney, P. K., & Gold, M. S. (1998). Immobility and flight associated with antinociception produced by activation of the ventral and lateral/dorsal regions of the rat periaqueductal grey. *Brain Research*, *804*, 159–166.

Morris, J. S., Frith, C. D., Perrett, D. I., Rowland, D., Young, A. W., Calder, A. J., et al. (1996). A differential neural response in the human amygdala to fearful and happy facial expressions. *Nature*, *6603*, 812–815.

Neophytou, S. I., Graham, M., Williams, J., Aspley, S., Marsden, C. A., & Beckett, S. R. G. (2000). Strain differences to the effects of aversive frequency ultrasound on behaviour and brain topography of c-fos expression in the rat. *Brain Research*, *854*, 158–164.

Nicolas, L. B., Klein, S., & Prinssen, E. P. (2007). Defensive-like behaviours induced by ultrasound: further pharmacological characterization in Lister-hooded rats. *Psychopharmacology*, *194*, 243–252.

Nobre, M. J., & Brandão, M. L. (2004). Analysis of freezing behavior and ultrasonic vocalization in response to foot-shock, ultrasound signals and GABAergic inhibition in the inferior colliculus: effects of muscimol and midazolam. *European Neuropsychopharmacology*, *14*, 45–52.

Ouda, L., Jilek, M., & Syka, J. (2016). Expression of c-Fos in rat auditory and limbic systems following 22-kHz calls. *Behavioural Brain Research*, *308*, 196–204.

Panksepp, J., & Burgdorf, J. (2003). "Laughing" rats and the evolutionary antecedents of human joy? *Physiology and Behavior*, *79*, 533–547.

Panksepp, J., Gordon, N., & Burgdorf, J. (2002). Empathy and the action-perception resonances of basic socio-emotional systems of the brain. *Behavioral Brain Sciences*, *25*, 43.

Panksepp, J., Siviy, S. M., & Normansell, L. A. (1984). The psychobiology of play: theoretical and methodological considerations. *Neuroscience and Biobehavioral Reviews*, *8*, 465–492.

Paré, W. P., Tejani-Butt, S., & Kluczynski, J. (2001). The emergence test: effects of psychotropic drugs on neophobic disposition in Wistar Kyoto (WKY) and Sprague Dawley rats. *Progress in Neuropsychopharmacology and Biological Psychiatry*, *25*, 1615–1628.

Parsana, A. J., Li, N., & Brown, T. H. (2012). Positive and negative ultrasonic social signals elicit opposing firing patterns in rat amygdala. *Behavioural Brain Research*, *226*, 77–86.

Parsana, A. J., Moran, E. E., & Brown, T. H. (2012). Rats learn to freeze to 22-kHz ultrasonic vocalizations through autoconditioning. *Behavioural Brain Research*, *232*, 395–399.

Pitkänen, A., Savander, V., & LeDoux, J. E. (1997). Organization of intra-amygdaloid circuitries in the rat: an emerging framework for understanding functions of the amygdale. *Trends in Neuroscience*, *20*, 517–523.

Pultorak, J. D., Kelm-Nelson, C. A., Holt, L. R., Blue, K. V., Ciucci, M. R., & Johnson, A. M. (2016). Decreased approach behavior and nucleus accumbens immediate early gene expression in response to Parkinsonian ultrasonic vocalizations in rats. *Social Neuroscience*, *11*, 365–379.

Sadananda, M., Wöhr, M., & Schwarting, R. K. W. (2008). Playback of 22-kHz and 50-kHz ultrasonic vocalizations induces differential c-fos expression in rat brain. *Neuroscience Letters*, *435*, 17–23.

Saito, Y., Yuki, S., Seki, Y., Kagawa, H., & Okanoya, K. (2016). Cognitive bias in rats evoked by ultrasonic vocalizations suggests emotional contagion. *Behavioural Processes*, *132*, 5–11.

Sales, G. D. (1972). Ultrasound and mating behaviour in rodents with some observations on other behavioural situations. *Journal of Zoology*, *168*, 149–164.

Sales, G. D. (1991). The effect of 22 kHz calls and artificial 38 kHz signals on activity in rats. *Behavioural Processes*, *24*, 83–93.

Schwarting, R. K. W., Jegan, N., & Wöhr, M. (2007). Situational factors, conditions and individual variables which can determine ultrasonic vocalizations in male adult Wistar rats. *Behavioural Brain Research*, *182*, 208–222.

Seffer, D., Rippberger, H., Schwarting, R. K. W., & Wöhr, M. (2015). Pro-social 50-kHz ultrasonic communication in rats: post-weaning but not post-adolescent social isolation leads to social impairments—phenotypic rescue by re-socialization. *Frontiers in Behavioral Neuroscience*, *9*, 102.

Seffer, D., Schwarting, R. K. W., & Wöhr, M. (2014). Pro-social ultrasonic communication in rats: insights from playback studies. *Journal of Neuroscience Methods*, *234*, 73–81.

Seligman, M. E. P. (1970). On the generality of the laws of learning. *Psychological Review*, *77*, 406–418.

Siviy, S. M., & Panksepp, J. (1987). Sensory modulation of juvenile play in rats. *Developmental Psychobiology*, *20*, 39–55.

Smith, L. K., Fantella, S. -L., & Pellis, S. M. (1999). Playful defensive responses in adult male rats depend upon the status of the unfamiliar opponent. *Aggressive Behavior*, *25*, 141–152.

Snoeren, E. M. S., & Agmo, A. (2013). Female ultrasonic vocalizations have no incentive value for male rats. *Behavioral Neuroscience*, *127*, 439–450.

Snoeren, E. M. S., & Agmo, A. (2014). The incentive value of males' 50-kHz ultrasonic vocalizations for female rats (*Rattus norvecigus*). *Journal of Comparative Psychology*, *128*, 40–55.

Thomas, D. A., & Barfield, R. J. (1985). Ultrasonic vocalization of the female rat (*Rattus norvegicus*) during mating. *Animal Behavior*, *33*, 720–725.

Thomas, D. A., Talalas, L., & Barfield, R. J. (1981). Effects of devocalization of the male on mating behavior in rats. *Journal of Comparative and Physiological Psychology*, *95*, 630–637.

Thompson, B., Leonard, K. C., & Brudzynski, S. B. (2006). Amphetamine-induced 50 kHz calls from rat nucleus accumbens: a quantitative mapping study and acoustic analysis. *Behavioural Brain Research*, *168*, 64–73.

Vanderschuren, L. J. M. J., RJM, N., & Van Ree, J. M. (1997). The neurobiology of social play behavior in rats. *Neuroscience and Biobehavioral Reviews*, *21*, 309–326.

Vianna, D. M. L., & Brandão, M. L. (2003). Anatomical connections of the periaqueductal gray: specific neural substrates for different kinds of fear. *Brazilian Journal of Medical and Biological Research*, *36*, 557–566.

Voits, M., Beckett, S. R. G., Marsden, C. A., & Fink, H. (1999). Role of cholecystokinin type B receptors in ultrasound induced behavior in rats. *Peptides*, *20*, 383–386.

White, N. R., & Barfield, R. J. (1987). Role of the ultrasonic vocalization of the female rat (*Rattus norvegicus*) in sexual behavior. *Journal of Comparative Psychology*, *101*, 73–81.

White, N. R., & Barfield, R. J. (1989). Playback of female rat ultrasonic vocalizations during sexual behavior. *Physiology and Behavior*, *45*, 229–233.

White, N. R., & Barfield, R. J. (1990). Effects of male pre-ejaculatory vocalizations on female receptive behavior in the rat (*Rattus norvegicus*). *Journal of Comparative Psychology*, *104*, 140–146.

White, N. R., Gonzales, R. N., & Barfield, R. J. (1993). Do vocalizations of the male rat elicit calling from the female? *Behavioral and Neural Biology*, *59*, 16–78.

Willadsen, M., Seffer, D., Schwarting, R. K. W., & Wöhr, M. (2014). Rodent ultrasonic communication: male pro-social 50-kHz ultrasonic vocalizations elicit social approach behavior in female rats (*Rattus norvegicus*). *Journal of Comparative Psychology*, *128*, 56–64.

Willuhn, I., Tose, A., Wanat, M. J., Hart, A. S., Hollon, N. G., Phillips, P. E. M., et al. (2014). Phasic dopamine release in the nucleus accumbens in response to pro-social 50-kHz ultrasonic vocalizations in rats. *The Journal of Neuroscience*, *34*, 10616–10623.

Wöhr, M., Borta, A., & Schwarting, R. K. W. (2005). Overt behavior and ultrasonic vocalization in a fear conditioning paradigm—a dose-response study in the rat. *Neurobiology of Learning and Memory*, *84*, 228–240.

Wöhr, M., Houx, B., Schwarting, R. K. W., & Spruijt, B. (2008). Effects of experience and context on 50-kHz vocalizations in rats. *Physiology and Behavior*, *93*, 766–776.

HBBN, 978-0-12-809600-0

B978-0-12-809600-0.00034-2

Wöhr, M., & Schwarting, R. K. W. (2007). Ultrasonic communication in rats: can playback of 50-kHz calls induce approach behavior? *PLoS One*, *2*, e1365.

Wöhr, M., & Schwarting, R. K. W. (2008a). Maternal care, isolation-induced infant ultrasonic calling, and their relations to adult anxiety-related behavior in the rat. *Behavioral Neuroscience*, *122*, 310–320.

Wöhr, M., & Schwarting, R. K. W. (2008b). Ultrasonic calling during fear conditioning in the rat: no evidence for an audience effect. *Animal Behavior*, *76*, 749–760.

Wöhr, M., & Schwarting, R. K. W. (2009). Ultrasonic communication in rats: effects of morphine and naloxone on vocal and behavioral responses to playback of 50-kHz vocalizations. *Pharmacology Biochemistry and Behavior*, *94*, 285–295.

Wöhr, M., & Schwarting, R. K. W. (2010). Activation of limbic system structures by replay of ultrasonic vocalization in rats. In S. M. Brudzynski (Ed.), *Handbook of Mammalian Vocalization. An Integrative Neuroscience Approach* (pp. 113–124). Amsterdam: Academic Press/Elsevier.

Wöhr, M., & Schwarting, R. K. W. (2012). Testing social acoustic memory in rats: effects of stimulus configuration and long-term memory on the induction of social approach behavior by appetitive 50-kHz ultrasonic vocalizations. *Neurobiology of Learning and Memory*, *98*, 154–164.

HBBN, 978-0-12-809600-0

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Abstract

Ultrasonic vocalizations in rats serve as important social signals, thereby receiving substantial scientific attention in behavioral and neurobiological research. To study their effects on call recipients, playback techniques were developed with the use of natural or artificial auditory signals that can be tested in terms of their effects on behavior and brain functions. The approach is also used to study the outcomes of psychopharmacological manipulations and their applicability in preclinical models of human disorders where social communicatory deficits are known to play a role. The present review summarizes the current scientific evidence for roles and effects of 22-kHz and 50-kHz ultrasonic vocalizations on juvenile and adult rats. These vocalizations represent two functionally opposite signal classes that serve aversive/defensive and affiliative/appetitive functions, respectively, and which are related to specific and partly distinct brain regions.

Keywords: Rat, Ultrasonic vocalization, Social communication, Alarm call, Contact call, Periaqueductal gray, Amygdala, Nucleus accumbens, Dopamine

Study I

Title: *Cacna1c* haploinsufficiency leads to pro-social 50-kHz ultrasonic communication deficits in rats

Authors: Theresa M. Kisko, Moria D. Braun, Susanne Michels, Stephanie H. Witt, Marcella Rietschel, Carsten Culmsee, Rainer K.W. Schwarting, Markus Wöhr

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Cacna1c haploinsufficiency leads to pro-social 50-kHz ultrasonic communication deficits in rats

Cacna1c and ultrasonic communication in rats

Original Research Article

Theresa M. Kisko[1]*, Moria D. Braun[1]*, Susanne Michels[2], Stephanie H. Witt[3], Marcella Rietschel[3], Carsten Culmsee[2,4], Rainer K.W. Schwarting[1,4], Markus Wöhr[1,4]

[1]Behavioral Neuroscience, Experimental and Biological Psychology, Faculty of Psychology, Philipps-University of Marburg, Gutenbergstr. 18, D-35032 Marburg, Germany
[2]Institute of Pharmacology and Clinical Pharmacy, Philipps-University of Marburg, Karl-von-Frisch-Str. 1, D-35032 Marburg, Germany
[3]Department of Genetic Epidemiology in Psychiatry, Central Institute of Mental Health, Faculty of Medicine Mannheim, University of Heidelberg, J5, D-65189 Mannheim, Germany
[4]Marburg Center for Mind, Brain, and Behavior (MCMBB)

* shared first authorship

Correspondence should be addressed to:
Markus Wöhr, PhD
ORCID: 0000-0001-6986-5684
Behavioral Neuroscience
Experimental and Biological Psychology
Philipps-University of Marburg
Gutenbergstr. 18, 35032 Marburg, Germany
Fax: +6421 28 23610, Tel: +6421 28 23612
e-mail: markus.woehr@staff.uni-marburg.de

ABSTRACT

The cross-disorder risk gene *CACNA1C* is strongly implicated in multiple neuropsychiatric disorders, including autism spectrum disorder (ASD), bipolar disorder (BPD), and schizophrenia (SCZ), with deficits in social functioning being common for all major neuropsychiatric disorders. In the present study, we explored the role of *Cacna1c* in regulating disorder-relevant behavioral phenotypes, focusing on socio-affective communication after weaning during the critical developmental period of adolescence in rats. To this aim, we used a newly developed genetic *Cacna1c* rat model and applied a truly reciprocal approach for studying communication through ultrasonic vocalizations, including both sender and receiver. Our results show that a deletion of *Cacna1c* leads to deficits in social behavior and pro-social 50-kHz ultrasonic communication in rats. Reduced levels of 50-kHz ultrasonic vocalizations emitted during rough-and-tumble play may suggest that *Cacna1c* haploinsufficient rats derive less reward from playful social interactions. Besides the emission of fewer 50-kHz ultrasonic vocalizations in the sender, *Cacna1c* deletion reduced social approach behavior elicited by playback of 50-kHz ultrasonic vocalizations. This indicates that *Cacna1c* haploinsufficiency has detrimental effects on 50-kHz ultrasonic communication in both, sender and receiver. Together, these data suggest that *Cacna1c* plays a prominent role in regulating socio-affective communication in rats with relevance for ASD, BPD, and SCZ.

Summary statement: The present study suggests that *Cacna1c* plays a prominent role in regulating socio-affective communication in rats with relevance for neuropsychiatric disorders.

Key words: $Ca_v1.2$, calcium, autism, social behavior, rough-and-tumble play, ultrasonic vocalizations

BACKGROUND

The cross-disorder risk gene *CACNA1C* is strongly implicated in multiple neuropsychiatric disorders, including autism spectrum disorder (ASD), bipolar disorder (BPD), and schizophrenia (SCZ) [Ferreira et al., 2008; Green et al., 2010; Nyegaard et al., 2010; Splawski et al., 2004]. *CACNA1C* codes for the α1C subunit of the voltage-gated L-type calcium channel (LTCC) $Ca_v1.2$, regulating depolarization-dependent calcium influx into the cell. $Ca_v1.2$ is accounting for the majority of all LTCCs in the brain. It plays a pivotal role in regulating neuronal excitability, synaptic plasticity, and gene expression, and thus represents a primary therapeutic target [Zamponi, 2016].

Deficits in social functioning, such as failure of normal back-and-forth conversation and abnormal social approach, are common for all major neuropsychiatric disorders [Meyer-Lindenberg and Tost, 2012] and genetic *Cacna1c* mouse models display prominent alterations in social behavior [Kabir et al., 2016]. While mice currently tend to be the most commonly used model species, rats have several advantages [Ellenbroek and Youn, 2016]. Benefits include genetic variability and overall behavioral richness, which may improve translational validity, particularly when it comes to studies on social behavior and communication [Homberg et al., 2017]. Rats are highly gregarious animals with a rich and complex social behavior repertoire. For instance, they display cooperation, reciprocity, and mutual reward preferences [Hernandez-Lallement et al., 2015], linked to empathy-driven helping behavior [Ben-Ami Bartal, 2011]. Importantly, rats begin interacting socially at very young age and juveniles engage in high levels of rough-and-tumble play behavior, making it the most used model species to study social play [Vanderschuren et al., 2016]. The complex nature of social play involves coordination and integration of behavior and communication, requiring numerous neural systems [Vanderschuren et al., 2016], and individual rough-and-tumble play components, such as pinning, wrestling, and chasing, were found to be selectively affected by genetic [Homberg et al., 2007], prenatal [Raza et al., 2015], pharmacological [Vanderschuren et al., 1995], and brain manipulations [Schneider and Koch, 2005].

Acoustic communication is another important component of their social behavior repertoire. Rats emit whistle-like calls in the ultrasonic range, i.e. ultrasonic vocalizations (USV; [Brudzynski, 2013]). Evidence from selective breeding, devocalization, and playback studies suggests that the various USV types serve as

situation-dependent socio-affective signals fulfilling distinct communicative functions. Specifically, 50-kHz USV are thought to reflect a positive affective state (“rat laughter”; [Panksepp, 2005]), as they occur in appetitive situations, most notably during and in anticipation of rough-and-tumble play [Knutson et al., 1998], and are required to maintain playful mood [Kisko et al., 2105]. They serve important pro-social communicative functions and 50-kHz USV playback induces social approach behavior in receivers by eliciting the anticipation of rewarding social interactions, suggesting that approach evoked by 50-kHz USV can be used as a behavioral readout for the incentive salience of social contact [Engelhardt et al., 2017].

MATERIALS AND METHODS

Animals and housing

Effects of *Cacna1c* haploinsufficiency on behavioral phenotypes with relevance for socio-affective communication deficits in ASD, BPD, and SCZ were assessed in male constitutive heterozygous *Cacna1c*$^{+/-}$ rats (N=20) and compared to wildtype *Cacna1c*$^{+/+}$ littermate controls (N=20). *Cacna1c*$^{+/-}$ rats were generated by means of zinc finger technology by SAGE Labs (now Horizon Discovery Ltd, Cambridge, UK) on a Sprague-Dawley (SD) background, following a previously established protocol [Geurts et al., 2009]. *Cacna1c*$^{+/-}$ rats carry a 4 base pair (bp) deletion at 460649-460652 bp in genomic sequence resulting in an early stop codon in exon 6. Homozygous *Cacna1c*$^{-/-}$ rats are embryonically lethal.

A heterozygous breeding protocol was used to obtain offspring from both genotypes. To this aim, SD females (Charles River, Sulzfeld, Germany) and male *Cacna1c*$^{+/-}$ rats were paired for breeding. SD females were used because breeding efficacy is reduced in female *Cacna1c*$^{+/-}$ rats. N=8 litters with N=16.25±0.67 pups were obtained, with equal sex (t_7=0.143, p=0.809) and genotype (t_7=0.540, p=0.606) ratios. In order to avoid litter effects, only litters with both genotypes present were included in the experiments. Breeding was performed at the Faculty of Psychology, Philipps-University of Marburg, Germany. Approximately 2 weeks after pairing for breeding, females were individually housed and inspected daily for pregnancy and delivery. The day of birth was considered as postnatal day (PND) 0. After weaning on PND 21, rats were socially housed in groups of 4-6 with same-sex littermate partners in polycarbonate Macrolon Type IV cages (Tecniplast Deutschland GmbH,

Hohenpeißenberg, Germany; 58 x 38 x 20 cm, length x width, x height) under standard laboratory conditions (22±2 °C and 40-70 % humidity) with free access to standard rodent chow and water. Rats were identified by paw tattoo, using non-toxic animal tattoo ink (Ketchum permanent tattoo inks green paste, Ketchum Manufacturing Inc., Brockville, Canada). The ink was inserted subcutaneously through a 30 gauge hypodermic needle tip into the center of the paw on PND 5±1.

Genotyping

Rat tail snips were collected by dissecting ~0.3 cm of tail on PND 5±1. Tails were digested, genomic DNA was isolated and purified using the Qiagen DNAeasy Blood & Tissue Kit according to the manufacturer's instructions (Hilden, Germany). After the extraction, 2.0 µl of DNA in buffer containing ~250-400 µg of DNA was amplified by PCR using the Promega PCR Master Mix (Mannheim, Germany). The following primers were used: GCTGCTGAGCCTTTTATTGG (*Cacna1c* Cel-1 F) and CCTCCTGGATAGCTGCTGAC (*Cacna1c* Cel-1 R). Genotyping was performed on a 3130xl Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA, USA).

Protein analysis

Protein extraction and Western blot were performed using frozen cortical tissue pieces (25-50 mg, left hemisphere) from 10 month old male *Cacna1c*$^{+/-}$ rats (N=6) and their *Cacna1c*$^{+/+}$ littermate controls (N=6). Each tissue sample was lysed in 600 µl buffer containing 50 mM Tris hydrochloride, 150 mM sodium chloride, 5 mM EDTA, 1 % (w/v) Triton X-100 and 0.5 % (w/v) sodium deoxycholate supplemented with protease and phosphatase inhibitor cocktail tablets (Roche Diagnostics, Mannheim, Germany) and homogenized with T10 basic Ultra-Turrax (IKA-Werke, Staufen, Germany) for 10 s. The homogenates were then centrifuged for 15 min at 13,000 xg and 4 °C (Heraeus Fresco^{TM} 17, Thermo Fisher Scientific, Darmstadt, Germany). The total protein amount was determined from the supernatants using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Darmstadt, Germany). Fifty µg protein per sample were loaded on a 7.5 % polyacrylamide gel. After electrophoresis, the proteins were transferred onto a PVDF membrane (Roche Diagnostics, Mannheim, Germany) and incubated with anti-Ca_V1.2 (1:500; Cat# ACC-003; Lot# ACC003AN5102; Alomone Labs, Jerusalem, Israel) and anti-Vinculin antibodies (1:20,000; Sigma-Aldrich,

München, Germany) overnight at 4 °C. Protein detection was realized using peroxidase labeled secondary antibodies (Vector Laboratories, Burlingame, CA, USA) and luminol based HRP-Juice Plus (PJK GmbH, Kleinblittersdorf, Germany). The resulting chemiluminescence was imaged with a ChemiDoc XRS system (Bio-Rad Laboratories, Hercules, CA, USA). Protein quantification was performed using Bio-Rad Image Lab™ Software. Unless otherwise stated, all reagents were purchased from Sigma-Aldrich (München, Germany).

Behavioral phenotyping

Behavioral phenotypes were assessed in male *Cacna1c*$^{+/-}$ rats and compared to *Cacna1c*$^{+/+}$ littermate controls by means of our established 50-kHz USV radial maze playback paradigm (PND 24±3), rough-and-tumble play behavior and pro-social 50-kHz USV (PND 32-34), as well as repetitive and stereotyped patterns of behavior (PND 64±3). All rats were tested in all three behavioral assays. Body weight did not differ between genotypes (for details, see Table S1; t_{38}=0.859, p=0.396; t_{18}=0.347, p=0.732 and t_{38}=0.166, p=0.869; respectively), in line with a lack of body weight differences and genotype effects on general health measures during early development, as assessed in an independent cohort of rats to avoid potential confounds due to repeated handling. Behavioral experiments were carried out during the light phase of a 12:12 h light / dark cycle (lights on at 06:00 h). Rats were handled for three consecutive days prior to behavioral testing in a standardized way for 5 min. Behavioral analysis was performed by an experienced observer blind to experimental condition.

Rough-and-tumble play and pro-social 50-kHz ultrasonic vocalizations

On PND 32-34, rough-and-tumble play behavior and the emission of pro-social 50-kHz USV were measured, using sample sizes and a modified protocol previously established [Lukas and Wöhr, 2015]. In rats, rough-and-tumble play behavior peaks around the age of PND 30-40 [Panksepp, 1981]. On three consecutive days, pairs of juvenile rats were allowed to socially interact for 5 min (referred to as play phase) in an, at first, unfamiliar observation arena (35 x 35 cm, with Plexiglas walls; floor covered with 1 cm of fresh bedding) after one rat of the pair being habituated to the test environment for 2 min (referred to as anticipation phase). A three days protocol was applied in order to assess changes in rough-and-tumble play and 50-kHz USV

emission induced by play experience, such as anticipatory 50-kHz USV [Knutson et al., 1998]. Rats were always paired with a same-sex, same-genotype, age-matched non-littermate and non-cagemate partner, since it is not yet possible to identify the sender of pro-social 50-kHz USV during rough-and-tumble play behavior in a reliable manner. To enhance the level of social motivation, subject rats were socially isolated for 24 h prior testing in a Makrolon type III cage (265 x 150 x 425 mm, plus high stainless-steel covers; Tecniplast Deutschland GmbH), and isolation was maintained throughout the three days testing period. For behavioral analyses, a digital camera (TK-1281 Color Video Camera, JVC, Yokohama, Japan) was used and connected to an external multimedia hard drive (ScreenPlay Pro HD, Iomega, San Diego, CA, USA). The following behavioral measures were scored by an experienced observer using The Observer XT (Noldus, Wagenigen, The Netherlands): duration of rough-and-tumble play, including pinning, wrestling, and chasing. Pinning was defined as one rat lying with its dorsal surface on the floor with the other rat standing over it. Wrestling was scored when a group of play-specific behaviors, including wrestling, boxing, and pouncing, occurred. Chasing was defined as moving in the direction of or pursuing the partner while the partner is moving away. Pro-social 50-kHz USV were recorded using an UltraSoundGate Condenser Microphone (CM16; Avisoft Bioacoustics, Berlin, Germany) placed 35 cm above the floor of the center of the observation arena. In an additional exploratory approach, detailed temporal analyses for linking individual playful events and 50-kHz USV were performed for the third play session by means of high-resolution ethograms using The Observer XT (Noldus, Wagenigen, The Netherlands). The generated composite ethograms representative for the first and third play session, respectively, were modified using a free and open source image editor, GIMP, with time reference, genotype, and play session being manually added. Notably, a red relative-time indicator used by The Observer XT and subsequently copied into the image export was removed, as it noticeably obscured data presentation. Rough-and-tumble play behavior and the emission of pro-social 50-kHz USV were measured under red light (~28 lux).

Playback of pro-social 50-kHz ultrasonic vocalizations

On PND 24±3, social approach behavior in response to pro-social 50-kHz USV was assessed on an elevated radial eight-arm maze (arms: 40.5 x 9.8 cm) under red light

(~10 lux) according to a modified playback protocol previously established [Seffer et al., 2015]. Particularly in males, social approach behavior induced by pro-social 50-kHz USV is clearly more prominent in juvenile than adult rats [Wöhr and Schwarting, 2007]. Acoustic stimuli were presented through an ultrasonic loudspeaker (ScanSpeak, Avisoft Bioacoustics) placed 20 cm away from the end of one arm. An additional, but inactive loudspeaker was arranged symmetrically at the opposite arm as a visual control. Two acoustic stimuli were used: (I) pro-social 50-kHz USV and (II) white noise; the latter serving as a time- and amplitude-matched acoustic stimulus control [Seffer et al., 2014]. Pro-social 50-kHz USV used for playback were recorded from a male rat during exploration of a cage containing scents from a recently separated cage mate. After an initial 15 min habituation period, each subject rat was exposed to 1 min playback presentations of 50-kHz USV and white noise, separated by a 10 min inter-stimulus interval. Stimulus order was counterbalanced to account for possible sequence effects. The session ended after an additional 10 min post-stimulus phase. Behavior was monitored by a video camera (Panasonic WV-BP 330/GE, Hamburg, Germany) mounted centrally above the arena. In response to 50-kHz USV and white noise playback, immediate head orientation was quantified. Total number of arm entries served as a measure for locomotor activity. Change values were calculated by subtracting the total number of arm entries per minute during the 5 minutes baseline period before playback from the total number of arm entries per minute during and after 50-kHz USV and white noise playback, respectively. Time spent on arms proximal and distal to the active ultrasonic loudspeaker was used to quantify approach and avoidance behavior, respectively. Change values were calculated by subtracting the time spent on proximal and distal arms per minute during the 5 minutes baseline period before playback from the time spent on proximal and distal arms per minute during and after 50-kHz USV playback. USV were monitored with two ultrasonic condenser microphones (CM16, Avisoft Bioacoustics) placed next to the loudspeakers.

Recording and analysis of ultrasonic vocalizations

UltraSoundGate Condenser CM16 Microphones (Avisoft Bioacoustics) sensitive to frequencies of 15–180 kHz (flat frequency response between 25 and 140 kHz; ±6 dB) were used for USV recordings. They were connected via an UltraSoundGate 416H USB audio device (Avisoft Bioacoustics) to a personal computer, where acoustic data

were recorded with a sampling rate of 250,000 Hz in 16-bit format (recording range: 0-125 kHz) by Avisoft RECORDER USGH. For acoustical analysis, recordings were transferred to Avisoft SASLab Pro (version 4.50). High resolution spectrograms (frequency resolution: 488 Hz; time resolution: 0.512 ms) were obtained through a fast Fourier transformation (512 FFT length, 100 % frame, Hamming window and 75 % time window overlap). Call detection of pro-social 50-kHz USV emitted by juvenile rats during rough-and-tumble play was provided by an experienced observer, who manually counted the numbers of USV in 20 s time bins. If two 50-kHz USV elements were at least 10 ms apart, two independent 50-kHz USV were counted. Based on previous studies on 50-kHz USV, additional parameters were determined for ~20,000 50-kHz USV emitted during the third play session, including call duration, peak frequency, frequency modulation, and peak amplitude [Wöhr et al., 2015]. Peak frequency and peak amplitude were derived from the average spectrum of the entire call. The extent of frequency modulation was defined as the difference between the lowest and the highest peak frequency within each call. Moreover, the 50-kHz USV profile was determined and 50-kHz USV emitted during the third play session were categorized into FLAT, STEP, TRILL, and MIXED 50-kHz USV subtypes using previously established [Pereira et al., 2014] and repeatedly applied criteria [Engelhardt et al., 2017; Wöhr et al., 2015]. Only rats emitting more than 5 calls per individual rough-and-tumble play component were included when comparing the prevalence of specific 50-kHz USV subtypes as percentages. In addition, the occurrence of ATYPICAL 50-kHz USV with comparatively low peak frequencies below 32 kHz and/or long call durations higher than 150 ms was determined. Finally, overlapping 50-kHz USV, i.e. when both rats were emitting 50-kHz USV at the same time, were included in the detailed spectrographic analysis.

Repetitive and stereotyped patterns of behavior

On PND 64±3, repetitive and stereotyped patterns of behavior were tested in a clean Makrolon type III cage (265 x 150 x 425 mm, plus high stainless-steel covers; Tecniplast Deutschland GmbH) without bedding material. For behavioral analyses, a digital camera (TK-1281 Color Video Camera, JVC) was used and connected to an external multimedia hard drive (ScreenPlay Pro HD, Iomega). Repetitive and stereotyped patterns of behavior were assessed by measuring the duration of self-

grooming and circling behavior during tail chasing. For assessing locomotor activity, the test cage was virtually divided in two halves by a line and the numbers of line crossings and rearing events were counted. Testing was performed under white light (~30 lux) conditions for 20 min.

Statistical Analysis

For comparing rough-and-tumble play behavior and pro-social 50-kHz USV between genotypes, analysis of variances (ANOVAs) for repeated measurements were calculated with the between-subject factor genotype (G) and the within-subject factors day (D), individual rough-and-tumble play components (C), and prevalence of specific 50-kHz USV subtypes (S), i.e. 50-kHz USV profiles. Playback of pro-social 50-kHz USV was analyzed using ANOVAs for repeated measurements with the between-subject factor genotype (G) and the within-subject factors time (T) and preference (P). Acoustic characteristics of 50-kHz USV, repetitive and stereotyped patterns of behavior, line crossings and rearing events, and Cav1.2 protein levels were compared between genotypes by means of unpaired t-tests. The chi^2-test was applied to compare immediate head orientation between genotypes. A p-value of <0.050 was considered statistically significant.

RESULTS

In the present study, we explored the role of *Cacna1c* in regulating behavioral phenotypes, focusing on socio-affective communication after weaning during the critical developmental period of adolescence in rats. To this aim, we used a newly developed genetic *Cacna1c* rat model and applied a truly reciprocal approach for studying communication through pro-social 50-kHz USV, including both sender and receiver. Effects of *Cacna1c* haploinsufficiency were assessed in male constitutive heterozygous *Cacna1c*$^{+/-}$ rats (N=20) and compared to wildtype *Cacna1c*$^{+/+}$ littermate controls (N=20). *Cacna1c*$^{+/-}$ rats were generated using zinc finger technology (for details, see materials and methods). As shown by western blot using cortical tissue, $Ca_V1.2$ protein levels of *Cacna1c*$^{+/-}$ rats are reduced by slightly more than 50% in the brain, as compared to *Cacna1c*$^{+/+}$ littermates ($t_{10}=4.345$, $p=0.001$; Figure 1; for representative Western blot and antibody validation, see Suppl. Figure S1).

Rough-and-tumble play and pro-social 50-kHz ultrasonic vocalizations

While *Cacna1c* haploinsufficiency did not lead to altered rough-and-tumble play behavior, concomitant emission of pro-social 50-kHz USV was strongly affected. Specifically, there were no genotype differences in play behavior with regards to time spent playing (G: $F_{1,18}$=0.037, p=0.849; Figure 2A) or individual playful events, i.e. pinning (G: $F_{1,18}$=0.045, p=0.835; Figure 2B), wrestling (G: $F_{1,18}$=0.046, p=0.833; Suppl. Figure S2A), and chasing (G: $F_{1,18}$=1.333, p=0.263; Suppl. Figure S2B). Across play sessions, the time engaged in playful social interactions increased, regardless of genotype (D: $F_{2,36}$=10.057, p<0.001; DxG: $F_{2,36}$=0.246, p=0.783). This was driven by a genotype-independent increase in pinning and wrestling duration (D: $F_{2,36}$=11.327, p<0.001; DxG: $F_{2,36}$=0.171, p=0.844 and D: $F_{2,36}$=10.748, p<0.001; DxG: $F_{2,36}$=0.412, p=0.666; respectively), while chasing did not change (D: $F_{2,36}$=0.671, p=0.518; DxG: $F_{2,36}$=1.672, p=0.202).

Despite unchanged rough-and-tumble play behavior, however, *Cacna1c*$^{+/-}$ rats emitted fewer 50-kHz USV than *Cacna1c*$^{+/+}$ littermates while engaged in playful encounters (G: $F_{1,18}$=5.648, p=0.029; Figure 2C). From the first play session, genotypes clearly differed, with prominent genotype effects being further evident during the second and third play session. During the anticipation phase, genotypes did not differ in 50-kHz USV emission (G: $F_{1,18}$=1.640, p=0.217). Irrespective of genotype, there was an increase in 50-kHz USV emission during anticipation (D: $F_{2,36}$=9.133, p=0.001; DxG: $F_{2,36}$=1.713, p=0.195) and during playful social interactions (D: $F_{2,36}$=22.546, p<0.001; DxG: $F_{2,36}$=0.319, p=0.729) across play session.

When performing detailed temporal analyses in an additional exploratory approach, specifically for the third play session, genotype differences in 50-kHz USV emission were found to be robust (G: $F_{1,18}$=16.159, p=0.009) and seen during play periods, i.e. while rats engaged in rough-and-tumble play behavior (t_{18}=2.352, p=0.030), but also during non-play periods (t_{18}=2.805, p=0.012; Figure 2D). Within play periods, 50-kHz USV levels differed between individual rough-and-tumble play components (C: $F_{2,36}$=16.159, p<0.001) and genotypes specifically during wrestling, with *Cacna1c*$^{+/-}$ rats emitting fewer 50-kHz USV than *Cacna1c*$^{+/+}$ littermates (t_{18}=2.529, p=0.021; Figure 2E). No genotype effects were evident during the other two playful events, i.e. pinning (t_{18}=0.290, p=0.775) and chasing (t_{18}=0.395, p=0.697; for representative ethograms: Figure 2F). In both *Cacna1c*$^{+/-}$ rats and *Cacna1c*$^{+/+}$ littermates, 50-kHz USV

emission was higher during play than non-play periods (t_9=3.021, p=0.014 and t_9=3.180, p=0.011; respectively), with particularly high 50-kHz USV emission rates during wrestling and chasing but not pinning in *Cacna1c*$^{+/+}$ littermates (pinning vs. wrestling: t_{19}=3.783, p=0.004; pinning vs. chasing: t_{19}=4.529, p=0.001; wrestling vs. chasing: t_{19}=0.438, p=0.672) and during chasing but not pinning and wrestling in *Cacna1c*$^{+/-}$ rats (pinning vs. wrestling: t_{19}=2.124, p=0.063; pinning vs. chasing: t_{19}=3.737, p=0.005; wrestling vs. chasing: t_{19}=2.370, p=0.042).

Moreover, differences in the prevalence of specific 50-kHz USV subtypes was evident (S: $F_{3,54}$=16.696, p<0.001), with the genotype difference in 50-kHz USV emission rates being driven by reduced FLAT and MIXED 50-kHz USV in *Cacna1c*$^{+/-}$ rats, as compared to *Cacna1c*$^{+/+}$ littermates (t_{18}=2.736, p=0.014 and t_{18}=3.420, p=0.003; respectively). STEP and TRILL 50-kHz USV were not affected by genotype (t_{18}=1.650, p=0.116 and t_{18}=0.295, p=0.771; respectively; Figure 3A). Importantly, genotype affected the 50-kHz USV profiles, i.e. the prevalence of specific 50-kHz USV subtypes, associated with individual rough-and-tumble play components (S: $F_{3,36}$=6.570, p=0.001; SxG: $F_{3,36}$=2.406, p=0.083; SxC: $F_{6,72}$=3.545, p=0.004; SxCxG: $F_{6,72}$=2.774, p=0.018; Figure 3B; for representative ethograms: Figure 3C). In *Cacna1c*$^{+/+}$ littermates, pinning was primarily associated with the occurrence of FLAT 50-kHz USV and, to a lesser extent, MIXED 50-kHz USV, while TRILL and STEP 50-kHz USV were rarely emitted. A similar pattern was obtained in *Cacna1c*$^{+/-}$ rats, with a large number of FLAT 50-kHz USV, moderate levels of MIXED and TRILL 50-kHz USV, but low rates of STEP 50-kHz USV. During wrestling, *Cacna1c*$^{+/+}$ littermates emitted high rates of MIXED and FLAT 50-kHz USV, together with moderate numbers of TRILL 50-kHz USV but low numbers of STEP 50-kHz USV. This was different in *Cacna1c*$^{+/-}$ rats, which produced a high number of TRILL and FLAT 50-kHz USV during wrestling but relatively low numbers of MIXED and particularly STEP 50-kHz USV. During chasing, high levels of MIXED 50-kHz USV, moderate rates of FLAT and TRILL 50-kHz USV, but low levels of STEP 50-kHz USV were evident in *Cacna1c*$^{+/+}$ littermates. In *Cacna1c*$^{+/-}$ rats, TRILL 50-kHz USV were most prominent, while FLAT, MIXED, and STEP 50-kHz USV did not occur often during chasing. In rare cases, both rats were emitting 50-kHz USV at the same time. The number of such overlapping 50-kHz USV did not differ between genotypes (t_{18}=1.472, p=0.158). Occasionally, ATYPICAL 50-kHz USV were detected at comparable levels in both genotypes (t_{18}=1.977, p=0.064).

Besides 50-kHz USV emission rates, acoustic characteristics of 50-kHz USV differed between genotypes. While call duration was not affected (t_{18}=0.987, p=0.337; Figure 4A), 50-kHz USV emitted by *Cacna1c*$^{+/-}$ rats were characterized by higher peak frequencies than the ones emitted by *Cacna1c*$^{+/+}$ littermates (t_{18}=2.677, p=0.015; Figure 4B), without differing in frequency modulation (t_{18}=0.259, p=0.799; Figure 4C). Moreover, 50-kHz USV emitted by *Cacna1c*$^{+/-}$ rats were lower in peak amplitude (t_{18}=3.330, p=0.004; Figure 4D). The increase in peak frequency seen in *Cacna1c*$^{+/-}$ rats was driven by a categorical shift in the relative occurrence of 50-kHz USV within two prominent call clusters. In both genotypes, two clusters were clearly evident. In the first cluster, 50-kHz USV are characterized by relatively low peak frequencies between 50 and 70 kHz. In the second cluster, 50-kHz USV are characterized by substantially higher peak frequencies between 70 and 90 kHz. *Cacna1c*$^{+/+}$ littermates emitted more low-frequency first cluster 50-kHz USV than high-frequency second cluster 50-kHz USV. Conversely, *Cacna1c*$^{+/-}$ rats emitted about the same number of first and second cluster 50-kHz USV, resulting in an overall increase in peak frequency. In contrast to peak frequency, the decrease in peak amplitude seen in *Cacna1c*$^{+/-}$ rats was due to a gradual reduction (Figure 4E).

Playback of pro-social 50-kHz ultrasonic vocalizations

Importantly, low emission of pro-social 50-kHz USV in the sender was paralleled by reduced responsivity to such 50-kHz USV in the receiver, with 50-kHz USV but not the acoustic control stimulus white noise (Figure 5A) leading to social approach behavior, as demonstrated by means of our established 50-kHz USV radial maze playback paradigm (Figure 5B). Specifically, the acoustic control stimulus white noise induced behavioral inhibition (T: $F_{1,38}$=104.143, p<0.001; TxG: $F_{1,38}$=0.134, p=0.717; Figure 5C). Both *Cacna1c*$^{+/-}$ rats and *Cacna1c*$^{+/+}$ littermates displayed reduced total arm entries during playback of white noise than before (T: $F_{1,19}$=101.605, p<0.001 and $F_{1,19}$=36.670, p<0.001; respectively). Moreover, behavioral inhibition was still evident after playback (T: $F_{1,38}$=127.529, p<0.001; TxG: $F_{1,38}$=0.009, p=0.927) and both genotypes continued to display reduced total arm entries after playback as compared to baseline (T: $F_{1,19}$=80.422, p<0.001 and $F_{1,19}$=52.123, p<0.001; respectively). No behavioral inhibition was seen in response to playback of 50-kHz USV. As compared to baseline before playback, during and after playback there was no change in total

arm entries, irrespective of genotype (T: $F_{1,38}$=0.122, p=0.728; TxG: $F_{1,38}$=0.005, p=0.945 and T: $F_{1,38}$=0.977, p=0.329; TxG: $F_{1,38}$=0.092, p=0.763; respectively). Of note, locomotor activity during the initial 15 min habituation period did not differ between genotypes, with total number of arm entries being similar in *Cacna1c*$^{+/-}$ rats and *Cacna1c*$^{+/+}$ littermates (G: $F_{1,38}$=1.119, p=0.297; TxG: $F_{14,532}$=1.270, p=0.222). Immediate head orientation in response to playback of 50-kHz USV and white noise was seen in almost all rats (~95%) and did not differ between genotypes (chi^2=2.105, p=0.147). There was no single rat not responding to both acoustic stimuli by head orientation.

Social approach behavior in response to playback of 50-kHz USV was reflected in a preference for arms proximal to the ultrasonic loudspeaker (T: $F_{1,38}$=50.904, p<0.001; P: $F_{1,38}$=68.242, p<0.001; TxP: $F_{1,38}$=103.775, p<0.001). This preference was strongly dependent on genotype (TxG: $F_{1,38}$=0.977, p=0.329; PxG: $F_{1,38}$=1.292, p=0.263; TxPxG: $F_{1,38}$=8.015, p=0.007; Figure 5D). Although both *Cacna1c*$^{+/-}$ rats and *Cacna1c*$^{+/+}$ littermates displayed social approach behavior and spent more time proximal during playback than before (T: $F_{1,19}$=23.608, p<0.001 and $F_{1,19}$=155.747, p<0.001; respectively), but less time distal (T: $F_{1,19}$=9.635, p=0.006 and $F_{1,19}$=32.618, p<0.001; respectively), resulting in a preference for proximal over distal arms in both genotypes (P: $F_{1,19}$=22.179, p<0.001 and $F_{1,19}$=108.615, p<0.001; respectively), the strength of the response was clearly genotype-dependent. In fact, the increase in time spent proximal was stronger in *Cacna1c*$^{+/+}$ than in *Cacna1c*$^{+/-}$ rats (t_{38}=2.561, p=0.015). Likewise, the reduction in time spent distal was stronger in *Cacna1c*$^{+/+}$ littermates (t_{38}=2.375, p=0.023). Similar genotype effects were evident in the minutes following 50-kHz USV playback (T: $F_{1,38}$=0.766, p=0.387; TxG: $F_{1,38}$=0.612, p=0.439; P: $F_{1,38}$=19.212, p<0.001; PxG: $F_{1,38}$=7.609, p=0.009; TxP: $F_{1,38}$=13.409, p=0.001; TxPxG: $F_{1,38}$=0.282, p=0.598). While *Cacna1c*$^{+/+}$ littermates continued displaying a preference for proximal over distal arms (P: $F_{1,19}$=15.721, p=0.001), no clear preference was evident in *Cacna1c*$^{+/-}$ rats (P: $F_{1,19}$=3.401, p=0.081). This was due to the fact that *Cacna1c*$^{+/+}$ littermates, but not *Cacna1c*$^{+/-}$ rats, kept spending more time proximal after playback than before (T: $F_{1,19}$=11.799, p=0.003 and $F_{1,19}$=2.607, p=0.123; respectively). They further kept spending less time distal (T: $F_{1,19}$=7.797, p=0.012 and $F_{1,19}$=2.635, p=0.121; respectively).

Besides the preference induced by 50-kHz USV playback, avoidance induced by the acoustic control stimulus white noise was modulated by genotype (T: $F_{1,38}$=3.773, p=0.060; TxG: $F_{1,38}$=0.085, p=0.772; P: $F_{1,38}$=5.421, p=0.025; PxG: $F_{1,38}$=11.467, p=0.002; TxP: $F_{1,38}$=4.885, p=0.033; TxPxG: $F_{1,38}$=1.289, p=0.263; Figure 5E). In fact, *Cacna1c*$^{+/+}$ littermates displayed clear avoidance of proximal arms (P: $F_{1,19}$=4.671, p=0.044), with the time spent on proximal arms being reduced during as compared to before playback (T: $F_{1,19}$=9.922, p=0.005) and the time spent on distal arms being unchanged (T: $F_{1,19}$=1.103, p=0.307). No such avoidance of proximal arms was evident in *Cacna1c*$^{+/-}$ rats (P: $F_{1,19}$=0.721, p=0.406), with the time spent on proximal and distal arms being unchanged (T: $F_{1,19}$=1.996, p=0.174 and $F_{1,19}$=0.090, p=0.767; respectively). A similar pattern was evident following white noise playback (T: $F_{1,38}$=2.776, p=0.104; TxG: $F_{1,38}$=1.672, p=0.204; P: $F_{1,38}$=8.358, p=0.006; PxG: $F_{1,38}$=13.943, p=0.001; TxP: $F_{1,38}$=4.959, p=0.032; TxPxG: $F_{1,38}$=2.106, p=0.155). Again, *Cacna1c*$^{+/+}$ littermate controls displayed clear avoidance of proximal arms (P: $F_{1,19}$=4.997, p=0.038), with reduced time spent on proximal arms (T: $F_{1,19}$=6.607, p=0.019) and unchanged time spent on distal arms (T: $F_{1,19}$=2.628, p=0.121). No avoidance was evident in *Cacna1c*$^{+/-}$ rats (P: $F_{1,19}$=0.465, p=0.503), with the time spent on proximal and distal arms being unchanged (T: $F_{1,19}$=2.152, p=0.159 and $F_{1,19}$=0.976, p=0.336; respectively).

Repetitive and stereotyped patterns of behavior

Finally, *Cacna1c* haploinsufficiency did not lead to enhanced levels of repetitive and stereotyped patterns of behavior, with tail chasing (t_{38}=0.211, p=0.834; Suppl. Figure S3A) and self-grooming (t_{38}=1.127, p=0.267; Suppl. Figure S3B) occurring at similar levels in both genotypes. Of note, locomotor activity during the assessment of repetitive and stereotyped patterns of behavior was not affected by genotype. Specifically, line crossings (t_{38}=1.538, p=0.132) and rearing events (t_{38}=1.517, p=0.137) occurred at similar levels in *Cacna1c*$^{+/-}$ rats and *Cacna1c*$^{+/+}$ littermates.

DISCUSSION

CACNA1C has emerged as a prime candidate susceptibility gene for neuropsychiatric disorders, particularly because single-nucleotide polymorphisms (SNPs) in *CACNA1C* rank among the most consistent and replicable findings from genome-wide association

studies [Cross-Disorder Group of the Psychiatric Genomics Consortium, 2013]. However, as rs1006737 and other identified SNPs are found in the intronic, i.e. the non-protein coding, region of *CACNA1C*, neurobiological mechanisms whereby such SNPs modify brain structure and function are not well understood. In fact, some reports have associated the risk variant rs1006737 with enhanced *CACNA1C* mRNA expression in post-mortem tissue and induced human neurons [Yoshimizu et al., 2015], whereas others reported decreased *CACNA1C* expression levels in the brains of SCZ and BPD patients carrying this risk allele [Gershon et al., 2014]. LTCC activity is also perturbed in a rare yet devastating disorder known as Timothy syndrome (TS) with features partly similar to ASD. Most cases arise from a G406R *CACNA1C* missense mutation [Splawski et al., 2004] and a TS mouse model carrying the G406R replacement in $Ca_v1.2$ was reported to display ASD-related behavioral phenotypes [Bader et al., 2011]. To our knowledge, however, behavioral phenotypes with relevance for socio-affective communication deficits in ASD, BPD, and SCZ have not been assessed in rats with genetic modifications targeting *Cacna1c* until now and available mouse studies almost exclusively focused on adult mice [Kabir et al., 2016], with no data being available on the role of *Cacna1c* in regulating socio-affective communication during the critical developmental period of adolescence.

Our results show for the first time that *Cacna1c* deletion leads to pro-social 50-kHz ultrasonic communication deficits and may suggest reduced incentive salience of social contact in *Cacna1c* haploinsufficient rats. While *Cacna1c* haploinsufficiency did not lead to altered rough-and-tumble play behavior, concomitant emission of 50-kHz USV was strongly affected. Over all three play sessions, *Cacna1c*$^{+/-}$ rats consistently emitted fewer 50-kHz USV while engaged in playful social interactions than *Cacna1c*$^{+/+}$ littermate controls. Genotype differences were evident during play and non-play periods, with *Cacna1c*$^{+/-}$ rats only reaching non-play period 50-kHz USV levels of *Cacna1c*$^{+/+}$ littermate controls during play periods. In an initial effort to link 50-kHz USV emission to specific individual playful events, we additionally showed for the first time, by means of temporal analyses using high-resolution ethograms, that wrestling and chasing are associated with particularly high 50-kHz USV rates in *Cacna1c*$^{+/+}$ littermate controls. Notably, this association was mild in *Cacna1c*$^{+/-}$ rats and low rates of 50-kHz USV were detected during wrestling. Within play periods, the genotype difference in 50-kHz USV was thus driven by reduced emission rates during wrestling but not

pinning or chasing. When performing a detailed spectrographic analysis, we further found that *Cacna1c* haploinsufficiency affected the 50-kHz USV profile by reducing FLAT and MIXED 50-kHz USV subtypes previously associated with the synchronization of complex social interactions [Łopuch and Popik, 2011]. Particularly during chasing, the prevalence of TRILL 50-kHz USV was enhanced in *Cacna1c*$^{+/-}$ rats at the expense of MIXED 50-kHz USV. Moreover, acoustic characteristics were found to be altered, with peak frequency being higher but peak amplitude being lower in *Cacna1c*$^{+/-}$ rats. This was at least in part due to alternative clustering. Together, since 50-kHz USV are believed to reflect positive affective states ("rat laughter"; [Panksepp, 2005]) associated with the rewarding nature of rough-and-tumble play [Vanderschuren et al., 2016], this suggests that *Cacna1c*$^{+/-}$ rats derive lower levels of reward from playful encounters, possibly due to impaired *liking* [Berridge et al., 2009].

Besides the emission of fewer 50-kHz USV in the sender, *Cacna1c* deletion reduced the behavioral responses elicited by 50-kHz USV playback, with social approach behavior clearly being more prominent in *Cacna1c*$^{+/+}$ littermate controls than in *Cacna1c*$^{+/-}$ rats. Importantly, genotype differences are unlikely due to auditory processing deficits. Immediate head orientation in response to playback of 50-kHz USV or white noise was seen in all rats and did not differ between genotypes. Moreover, both genotypes displayed behavioral inhibition when exposed to white noise playback, with the strength of the response not differing between genotypes. However, *Cacna1c*$^{+/+}$ littermate controls, but not *Cacna1c*$^{+/-}$ rats, further displayed clear avoidance behavior and moved away from the sound source in response to white noise playback. The avoidance response displayed by *Cacna1c*$^{+/+}$ littermate controls was long-lasting and still seen in the minutes following playback. Lack of avoidance in *Cacna1c*$^{+/-}$ rats might appear surprising given the ample evidence for increased anxiety-related behavior in constitutive *Cacna1c* heterozygous mice [Lee et al., 2012], particularly in females [Dao et al., 2010], yet strong behavioral inhibition seen in both genotypes speaks for alterations in coping strategies rather than anxiety levels. Finally, genotype differences in social approach behavior in response to 50-kHz USV playback were not due to impairments in behavioral activity and motor functions. Locomotor activity and rearing behavior did not differ between genotypes. Together, this suggests that genotype differences in social approach behavior evoked by 50-kHz USV reflects genotype effects on the motivation, i.e. *wanting*, for social contact, which is expressed

in the amount of effort spent to obtain a social reward [Berridge et al., 2009]. Notably, the observed deficits in social approach behavior in response to 50-kHz ultrasonic vocalizations are more prominent in our newly developed rat model than in a well-established *Shank3* rat model for ASD [Berg et al., in press], emphasizing the severity of the social deficits displayed by *Cacna1c* haploinsufficient rats. Together with the reduced 50-kHz USV emission rates during playful social interactions, this may, therefore, suggest deficits in *wanting* in addition to the *liking* component associated with playful encounters. Interestingly, reward processing and 50-kHz ultrasonic communication are both linked to dopamine [Burgdorf et al., 2007]. Thus, 50-kHz USV playback evokes phasic dopamine release in the nucleus accumbens [Willuhn et al., 2014] and dopamine signaling is profoundly altered in genetic *Cacna1c* mouse models [Terrillion et al., 2017a].

Our results indicate that a deletion of *Cacna1c* leads to deficits in social behavior and pro-social 50-kHz ultrasonic communication in rats. This is at least partially in line with currently available mouse studies. Traditionally, social behavior in mouse models is assessed using the three-chambered social approach assay, with intact sociability being defined as spending more time in proximity to a conspecific over an empty corral [Silverman et al., 2010]. Using this classic assay, Kabir et al. [2017] and Dedic et al. [2018] found that adult forebrain *Cacna1c* null mutant mice do not show a preference for the conspecific. Lack of sociability was also seen after *Cacna1c* knockdown specifically in the prefrontal cortex [Kabir et al., 2017], but not the nucleus accumbens [Terrillion et al., 2017b]. Moreover, in a modified version of the task, a mild reduction in sociability was seen in the TS mouse model carrying the G406R replacement in $Ca_v1.2$ [Bader et al., 2011] (but see [Kabitzke et al., 2018]), although this is a gain-of-function mutation in $Ca_v1.2$ characterized by reduced inactivation [Splawski et al., 2004]. Further evidence for a role of *Cacna1c* in regulating socio-affective communication comes from a study by Jeon et al. [2010], who showed that observational fear learning in mice is impaired following local $Ca_v1.2$ deletion in the anterior cingulate cortex. However, in constitutive *Cacna1c* heterozygous mice, no evidence for social deficits was obtained in two independent studies [Bader et al., 2011; Dedic et al., 2018] (for a comprehensive overview on the behavioral effects of genetic modifications targeting *Cacna1c* in mice, see [Kabir et al., 2016]). The fact that social deficits were only evident in *Cacna1c* null mutant but not *Cacna1c* heterozygous mice,

589 while in rats *Cacna1c* haploinsufficiency already results in deficits, is possibly due to
590 the richer social behavior repertoire of rats, with pro-social 50-kHz USV being
591 particularly sensitive for detecting disorder-relevant behavioral phenotypes.
592 In summary, reduced levels of 50-kHz USV emitted during rough-and-tumble play may
593 suggest that *Cacna1c* haploinsufficient rats derive less reward from playful social
594 interactions. Besides the emission of fewer 50-kHz USV in the sender, *Cacna1c*
595 deletion reduced the behavioral responses elicited by 50-kHz USV playback. This
596 indicates that *Cacna1c* haploinsufficiency has detrimental effects on 50-kHz ultrasonic
597 communication in both, sender and receiver. Together, these data suggest that
598 *Cacna1c* plays a prominent role in regulating socio-affective communication in rats with
599 relevance for ASD, BPD, and SCZ.

DECLARATIONS

List of Abbreviations: bipolar disorder (BPD); major depressive disorder (MDD); schizophrenia (SCZ); autism spectrum disorder (ASD); voltage-gated L-type calcium channel (LTCC); single-nucleotide polymorphism (SNP); genome-wide association study (GWAS); heterozygous *Cacna1c* (*Cacna1c*$^{+/-}$); wildtype *Cacna1c* (*Cacna1c*$^{+/+}$); postnatal day (PND), Sprague-Dawley (SD), ultrasonic vocalizations (USV).

Ethics Approval: All procedures were conducted in strict accordance with the the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and the relevant local or national rules and regulations of Germany and were subject to prior authorization by the local government (MR 20/35 Nr. 19/2014; Tierschutzbehörde, Regierungspräsdium Gießen, Germany).

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Authors contributions: MW conceived the study; TK, MB, and SM performed the experiments; TK and MW analyzed the data; TK and MW wrote the manuscript with the help of all other authors; SW, MR, CC, RS, and MW acquired funding.

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REFERENCES

Bader, P.L., Faizi, M., Kim, L.H., Owen, S.F., Tadross, M.R., Alfa, R.W., et al. (2011). Mouse model of Timothy syndrome recapitulates triad of autistic traits. *Proc. Natl. Acad. Sci. U. S. A.* **108**, 15432-15437.

Ben-Ami Bartal, I., Decety, J. and Mason, P. (2011). Empathy and pro-social behavior in rats. *Science.* **334**, 1427-1430.

Berg, E.L., Copping, N.A., Rivera, J.K., Pride, M.C., Careaga, M., Bauman, M.D., et al. (2018). Developmental social communication deficits in the Shank3 rat model of Phelan-McDermid syndrome and autism spectrum disorder. *Autism Res.* in press.

Berridge, K.C., Robinson, T.E. and Aldridge, J.W. (2009). Dissecting components of reward: 'liking', 'wanting', and learning. *Curr. Opin. Pharmacol.* **9**, 65-73.

Brudzynski, S.M. (2013). Ethotransmission: communication of emotional states through ultrasonic vocalization in rats. *Curr. Opin. Neurobiol.* **23**, 310-317.

Burgdorf, J., Wood, P.L., Kroes, R.A., Moskal, J.R. and Panksepp, J. (2007). Neurobiology of 50-kHz ultrasonic vocalizations in rats: Electrode mapping, lesion, and pharmacology studies. *Behav. Brain Res.* **182**, 274-283.

Cross-Disorder Group of the Psychiatric Genomics Consortium. (2013). Identification of risk loci with shared effects on five major psychiatric disorders: a genome-wide analysis. *Lancet.* **381**, 1371-1379.

Dao, D.T., Mahon, P.B., Cai, X., Kovacsics, C.E., Blackwell, R.A., Arad, M., et al. (2010). Mood disorder susceptibility gene CACNA1C modifies mood-related behaviors in mice and interacts with sex to influence behavior in mice and diagnosis in humans. *Biol. Psychiatry.* **68**, 801-810.

Dedic, N., Pöhlmann, M.L., Richter, J.S., Mehta, D., Czamara, D., Metzger, M.W., et al. (2018). Cross-disorder risk gene CACNA1C differentially modulates susceptibility to psychiatric disorders during development and adulthood. *Mol. Psychiatry.* **23**, 533-543.

Ellenbroek, B. and Youn, J. (2016). Rodent models in neuroscience research: is it a rat race? *Dis. Model Mech.* **9**, 1079-1087.

Engelhardt, K.A., Fuchs, E., Schwarting, R.K.W. and Wöhr, M. (2017). Effects of amphetamine on pro-social ultrasonic communication in juvenile rats: Implications for mania models. *Eur. Neuropsychopharmacol.* **27**, 261-273.

Ferreira, M.A., O'Donovan, M.C., Meng, Y.A., Jones, I.R., Ruderfer, D.M., Jones, L., et al. (2008). Collaborative genome-wide association analysis supports a role for ANK3 and CACNA1C in bipolar disorder. *Nat. Genet.* **40**, 1056-1058.

Gershon, E.S., Grennan, K., Busnello, J., Badner, J.A., Ovsiew, F., Memon, S., et al. (2014). A rare mutation of CACNA1C in a patient with bipolar disorder, and decreased gene expression associated with a bipolar-associated common SNP of CACNA1C in brain. *Mol. Psychiatry.* **19**, 890-894.

Geurts, A.M., Cost, G.J., Freyvert, Y., Zeitler, B., Miller, J.C., Choi, V.M., et al. (2009). Knockout rats via embryo microinjection of zinc-finger nucleases. *Science.* **325**, 433.

Green, E.K., Grozeva, D., Jones, I., Jones, L., Kirov, G., Caesar, S., et al. (2010). The bipolar disorder risk allele at CACNA1C also confers risk of recurrent major depression and of schizophrenia. *Mol. Psychiatry.* **15**, 1016-1022.

Hernandez-Lallement, J., Van Wingerden, M., Marx, C., Srejic, M. and Kalenscher, T. (2015). Rats prefer mutual rewards in a prosocial choice task. *Front. Neurosci.* **8**, 443.

Homberg, J.R., Schiepers, O.J., Schoffelmeer, A.N., Cuppen, E. and Vanderschuren, L.J. (2007). Acute and constitutive increases in central serotonin levels reduce social play behaviour in peri-adolescent rats. *Psychopharmacology (Berl).* **195**, 175-182.

Homberg, J.R., Wöhr, M. and Alenina, N. (2017). Comeback of the rat in biomedical research. *ACS Chem. Neurosci.* **8**, 900-903.

Jeon, D., Kim, S., Chetana, M., Jo, D., Ruley, H.E., Lin, S.-Y., et al. (2010). Observational fear learning involves affective pain system and Cav1.2 Ca2+ channels in ACC. *Nat. Neurosci.* **13**, 482-488.

Kabir, Z.D., Che, A., Fischer, D.K., Rice, R.C., Rizzo, B.K., Byrne, M., et al. (2017). Rescue of impaired sociability and anxiety-like behavior in adult cacna1c-deficient mice by pharmacologically targeting eIF2α. *Mol. Psychiatry.* **22**, 1096-1109.

Kabir, Z.D., Lee, A.S. and Rajadhyaksha, A.M. (2016). L-type Ca2+ channels in mood, cognition and addiction: integrating human and rodent studies with a focus on behavioural endophenotypes. *J. Physiol.* **594**, 5823-5837.

Kabitzke, P., Brunner, D., He, D., Fazio, P.A., Cox, K., Sutphen, J., et al. (2018). Comprehensive analysis of two Shank3 and the Cacna1c mouse models of autism spectrum disorder. *Genes Brain Behav.* **17**, 4-22.

Kisko, T.M., Himmler, B.T., Himmler, S.M., Euston, D.R. and Pellis, S.M. (2015). Are 50-kHz calls used as play signals in the playful interactions of rats? II. Evidence from the effects of devocalization. *Behav. Processes.* **111**, 25-33.

Knutson, B., Burgdorf, J. and Panksepp, J. (1998). Anticipation of play elicits high-frequency ultrasonic vocalizations in young rats. *J. Comp. Psychol.* **112**, 65-73.

Lee, A.S., Ra, S., Rajadhyaksha, A.M., Britt, J.K., De Jesus-Cortes, H., Gonzales, K.L., et al. (2012). Forebrain elimination of cacna1c mediates anxiety-like behavior in mice. *Mol. Psychiatry.* **17**, 1054-1055.

Łopuch, S. and Popik, P. (2011). Cooperative behavior of laboratory rats (Rattus norvegicus) in an instrumental task. *J. Comp. Psychol.* **125**, 250-253.

Lukas, M. and Wöhr, M. (2015). Endogenous vasopressin, innate anxiety, and the emission of pro-social 50-kHz ultrasonic vocalizations during social play behavior in juvenile rats. *Psychoneuroendocrinology.* **56**, 35-44.

Meyer-Lindenberg, A. and Tost, H. (2012). Neural mechanisms of social risk for psychiatric disorders. *Nat. Neurosci.* **15**, 663-668.

Nyegaard, M., Demontis, D., Foldager, L., Hedemand, A., Flint, T.J., Sørensen, K.M., et al. (2010). CACNA1C (rs1006737) is associated with schizophrenia. *Mol. Psychiatry.* **15**, 119-121.

Panksepp, J. (1981). The ontogeny of play in rats. *Dev. Psychobiol.* **14**, 327-332.

Panksepp, J. (2005). Psychology. Beyond a joke: from animal laughter to human joy? *Science.* **308**, 62-63.

Pereira, M., Andreatini, R., Schwarting, R.K.W. and Brenes, J.C. (2014). Amphetamine-induced appetitive 50-kHz calls in rats: a marker of affect in mania? *Psychopharmacology (Berl).* **231**, 2567-2577.

Raza, S., Himmler, B.T., Himmler, S.M., Harker, A., Kolb, B., Pellis, S.M. and Gibb, R. (2015). Effects of prenatal exposure to valproic acid on the development of juvenile-typical social play in rats. *Behav. Pharmacol.* **26**, 707-719.

Schneider, M. and Koch, M. (2005). Deficient social and play behavior in juvenile and adult rats after neonatal cortical lesion: effects of chronic pubertal cannabinoid

treatment. *Neuropsychopharmacology.* **30**, 944-957.

Seffer, D., Rippberger, H., Schwarting, R.K.W. and Wöhr, M. (2015). Pro-social 50-kHz ultrasonic communication in rats: post-weaning but not post-adolescent social isolation leads to social impairments-phenotypic rescue by re-socialization. *Front. Behav. Neurosci.* **9**, 102.

Seffer, D., Schwarting, R.K.W. and Wöhr, M. (2014). Pro-social ultrasonic communication in rats: insights from playback studies. *J. Neurosci. Methods.* **234**, 73-81.

Silverman, J.L., Yang, M., Lord, C. and Crawley, J.N. (2010). Behavioural phenotyping assays for mouse models of autism. *Nat. Rev. Neurosci.* **11**, 490-502.

Splawski, I., Timothy, K.W., Sharpe, L.M., Decher, N., Kumar, P., Bloise, R., et al. (2004). Ca(V)1.2 calcium channel dysfunction causes a multisystem disorder including arrhythmia and autism. *Cell.* **119**, 19-31.

Terrillion, C.E., Dao, D.T., Cachope, R., Lobo, M.K., Puche, A.C., Cheer, J.F. and Gould, T.D. (2017a). Reduced levels of Cacna1c attenuate mesolimbic dopamine system function. *Genes Brain Behav.* **16**, 495-505.

Terrillion, C.E., Francis, T.C., Puche, A.C., Lobo, M.K. and Gould, T.D. (2017b). Decreased nucleus accumbens expression of psychiatric disorder risk gene Cacna1c promotes susceptibility to social stress. *Int. J. Neuropsychopharmacol.* **20**, 428-433.

Vanderschuren, L.J., Achterberg, E.J. and Trezza, V. (2016). The neurobiology of social play and its rewarding value in rats. *Neurosci. Biobehav. Rev.* **70**, 86-105.

Vanderschuren, L.J., Niesink, R.J., Spruijt, B.M. and Van Ree, J.M. (1995). Effects of morphine on different aspects of social play in juvenile rats. *Psychopharmacology (Berl).* **117**, 225-231.

Willuhn, I., Tose, A., Wanat, M.J., Hart, A.S., Hollon, N.G., Phillips, P.E., et al. (2014). Phasic dopamine release in the nucleus accumbens in response to pro-social 50 kHz ultrasonic vocalizations in rats. *J. Neurosci.* **34**, 10616-10623.

Wöhr, M., Rippberger, H., Schwarting, R.K.W. and van Gaalen, M.M. (2015) Critical involvement of 5-HT2C receptor function in amphetamine-induced 50-kHz ultrasonic vocalizations in rats. *Psychopharmacology (Berl).* **232**, 1817-1829.

Wöhr, M. and Schwarting, R.K.W. (2007) Ultrasonic communication in rats: can playback of 50-kHz calls induce approach behavior? *PLoS One.* **2**, 1365.

Yoshimizu, T., Pan, J.Q., Mungenast, A.E., Madison, J.M., Su, S., Ketterman, J., et al. (2015). Functional implications of a psychiatric risk variant within CACNA1C in induced human neurons. *Mol. Psychiatry.* **20**, 162-169.

Zamponi, G.W. (2016). Targeting voltage-gated calcium channels in neurological and psychiatric diseases. *Nat. Rev. Drug Discov.* **15**, 19-34.

Figure Legends

Figure 1: $Ca_V1.2$ protein levels in *Cacna1c*$^{+/-}$ rats and *Cacna1c*$^{+/+}$ littermate controls. $Ca_V1.2$ expression levels were analyzed by Western blot from cortical tissue of male *Cacna1c*$^{+/-}$ rats (white bars; N=6) and *Cacna1c*$^{+/+}$ littermate controls (black bars; N=6). The bar graphs (left panel) were obtained by densitometric quantification of the Western blot data. The results are expressed as percentage of *Cacna1c*$^{+/+}$ littermate control values after normalization to the loading control vinculin. The $Ca_V1.2$ level of *Cacna1c*$^{+/+}$ littermate controls is set as 100 %. The immunoblots (right panel) show one representative example per genotype. Data are presented as mean±SEM. * $p<0.050$ vs. *Cacna1c*$^{+/+}$ littermate controls.

Figure 2: Rough-and-tumble play behavior and concomitant 50-kHz USV emission in *Cacna1c*$^{+/-}$ rats and *Cacna1c*$^{+/+}$ littermate controls. (**A**) Time spent playing; (**B**) time spent pinning; and (**C**) 50-kHz USV emission across the three play sessions in male *Cacna1c*$^{+/-}$ rats (white circles; N=10) and *Cacna1c*$^{+/+}$ littermate controls (black circles; N=10). (**D**) 50-kHz USV emission during play versus non-play phases and (**E**) during individual play events in male *Cacna1c*$^{+/-}$ rats (white circles; N=10) and *Cacna1c*$^{+/+}$ littermate controls (black circles; N=10), with 50-kHz USV being presented relative to the duration of play versus no-play phases and individual play events. (**F**) Representative, composite, and consolidated ethograms of a *Cacna1c*$^{+/-}$ rat pair (upper panels) and a *Cacna1c*$^{+/+}$ littermate control pair (lower panels) of the first and third play session, respectively. Pinning (blue), wrestling (green), and chasing (brown) events are depicted, together with 50-kHz USV (red) for the entire 5 min play sessions. Data are presented as mean±SEM. $^{\#}$ $p<0.050$ vs. first play session (in A, B, C), vs. play (in D), vs. pinning or wrestling (in E); * $p<0.050$ vs. *Cacna1c*$^{+/+}$ littermate controls.

Figure 3: Subtypes of pro-social 50-kHz USV emitted by *Cacna1c*$^{+/-}$ rats and *Cacna1c*$^{+/+}$ littermate controls during rough-and-tumble play behavior. (**A**) Emission of the different 50-kHz USV subtypes, i.e. FLAT, STEP, TRILL, and MIXED 50-kHz USV, in male *Cacna1c*$^{+/-}$ rats (white circles; N=10) and *Cacna1c*$^{+/+}$ littermate controls (black circles; N=10) during the third play session. (**B**) Pie charts depicting the

proportion of the different 50-kHz USV subtypes emitted by male *Cacna1c*$^{+/-}$ rats (lower panel) and *Cacna1c*$^{+/+}$ littermate controls (upper panel) during individual play events, i.e. pinning, wrestling, and chasing, of the third play session. The proportion of FLAT, STEP, TRILL, and MIXED 50-kHz USV is shown in black, dark gray, light gray, and white, respectively. (**C**) Detailed representative, composite, and consolidated ethograms of a *Cacna1c*$^{+/-}$ rat pair (lower panel) and a *Cacna1c*$^{+/+}$ littermate control pair (upper panel) of the third play session. Pinning (blue), wrestling (green), and chasing (brown) events are depicted, together with the 50-kHz USV subtypes (modified to reflect order in text), i.e. FLAT (red), STEP (yellow), TRILL (turquoise), and MIXED (purple), for 10 s of the entire 5 min play sessions. Data are presented as mean±SEM. * $p<0.050$ vs. *Cacna1c*$^{+/+}$ littermate controls.

Figure 4: Acoustic characteristics of pro-social 50-kHz USV emitted by *Cacna1c*$^{+/-}$ rats and *Cacna1c*$^{+/+}$ littermate controls during rough-and-tumble play behavior. (**A**) Call duration (in milliseconds [ms]); (**B**) peak frequency (in kilohertz [kHz]); (**C**) frequency modulation (in kilohertz [kHz]); and (**D**) peak amplitude (in decibel [dB]) of 50-kHz USV emitted by male *Cacna1c*$^{+/-}$ rats (white bars and grey frequency histograms; N=10) and *Cacna1c*$^{+/+}$ littermate controls (black bars and black frequency histograms; N=10) during the third play session. (**E**) Density plots depicting the distribution of individual 50-kHz USV depending on peak frequency (in kilohertz [kHz]) and peak amplitude (in decibel [dB]) emitted by male *Cacna1c*$^{+/-}$ rats (+/-; N=10) and *Cacna1c*$^{+/+}$ littermate controls (+/+; N=10) during the third play session. Color coding reflects frequencies as percentages. Density plots were generated by including more than 8,000 50-kHz USV emitted by male *Cacna1c*$^{+/-}$ rats and more than 10,000 50-kHz USV emitted by *Cacna1c*$^{+/+}$ littermate controls. Data are presented as mean±SEM. * $p<0.050$ vs. *Cacna1c*$^{+/+}$ littermate controls.

Figure 5: Social approach behavior evoked by pro-social 50-kHz USV playback in *Cacna1c*$^{+/-}$ rats and *Cacna1c*$^{+/+}$ littermate controls. (**A**) Exemplary spectrograms of acoustic stimuli used for playback, namely pro-social 50-kHz USV (upper panel) and time- and amplitude-matched white noise (lower panel). (**B**) Schematic illustration of the radial maze used for playback depicting proximal (black), distal (grey), and neutral (white) arms relative to the active ultrasonic loudspeaker. (**C**) Change in locomotor

activity in male *Cacna1c*$^{+/-}$ rats (white bars; N=20) and *Cacna1c*$^{+/+}$ littermate controls (black bars; N=20) as measured by total arm entries per minute during (left) and after (right) 50-kHz USV and white noise playback, as compared to the 5 minutes baseline period before playback. (**D**) Change in social approach behavior in male *Cacna1c*$^{+/-}$ rats (white bars; N=20) and *Cacna1c*$^{+/+}$ littermate controls (black bars; N=20) as measured by time spent on proximal (PROX) and distal (DIST) arms per minute during (left) and after (right) 50-kHz USV playback, as compared to the 5 minutes baseline period before playback. (E) Change in avoidance behavior in male *Cacna1c*$^{+/-}$ rats (white bars; N=20) and *Cacna1c*$^{+/+}$ littermate controls (black bars; N=20) as measured by time spent on proximal (PROX) and distal (DIST) arms per minute during (left) and after (right) white noise playback, as compared to the 5 minutes baseline period before playback. The dashed line represents baseline levels. Data are presented as mean±SEM. $^{\#}$ $p<0.050$ vs. baseline levels; ^{x} $p<0.050$ vs. distal; * $p<0.050$ vs. *Cacna1c*$^{+/+}$ littermate controls.

FIGURE 1

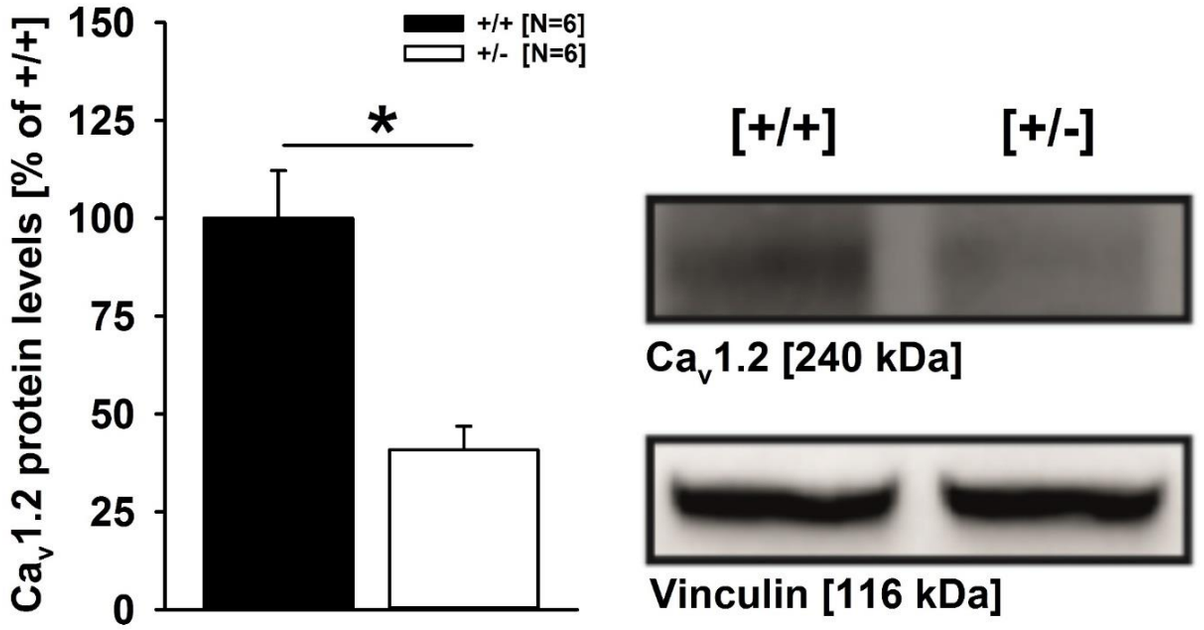

+/+ [N=6]
+/- [N=6]
$Ca_v1.2$ protein levels [% of +/+]
150
125
100
75
50
25
0
*
[+/+]
[+/-]
$Ca_v1.2$ [240 kDa]
Vinculin [116 kDa]

FIGURE 2

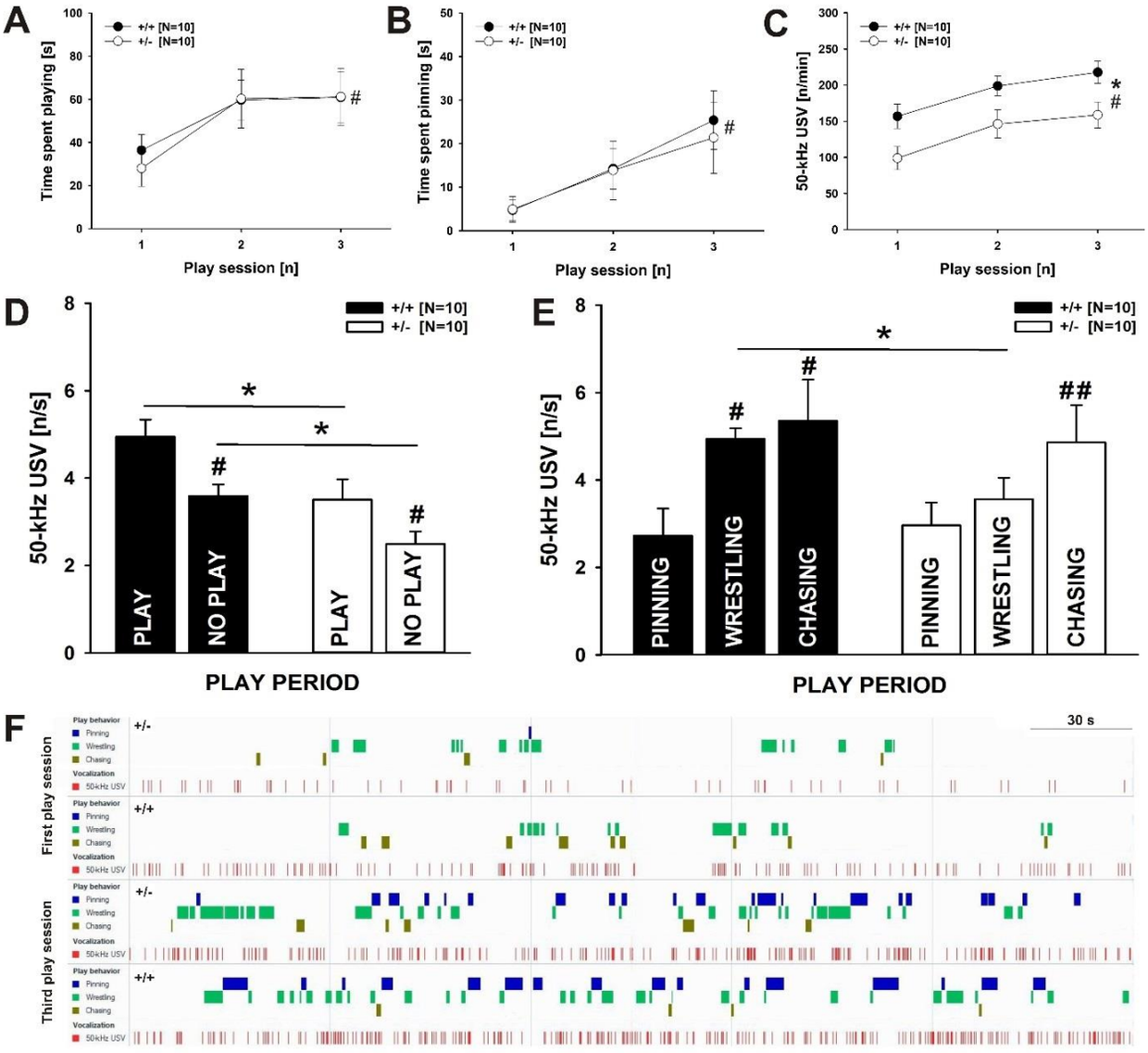
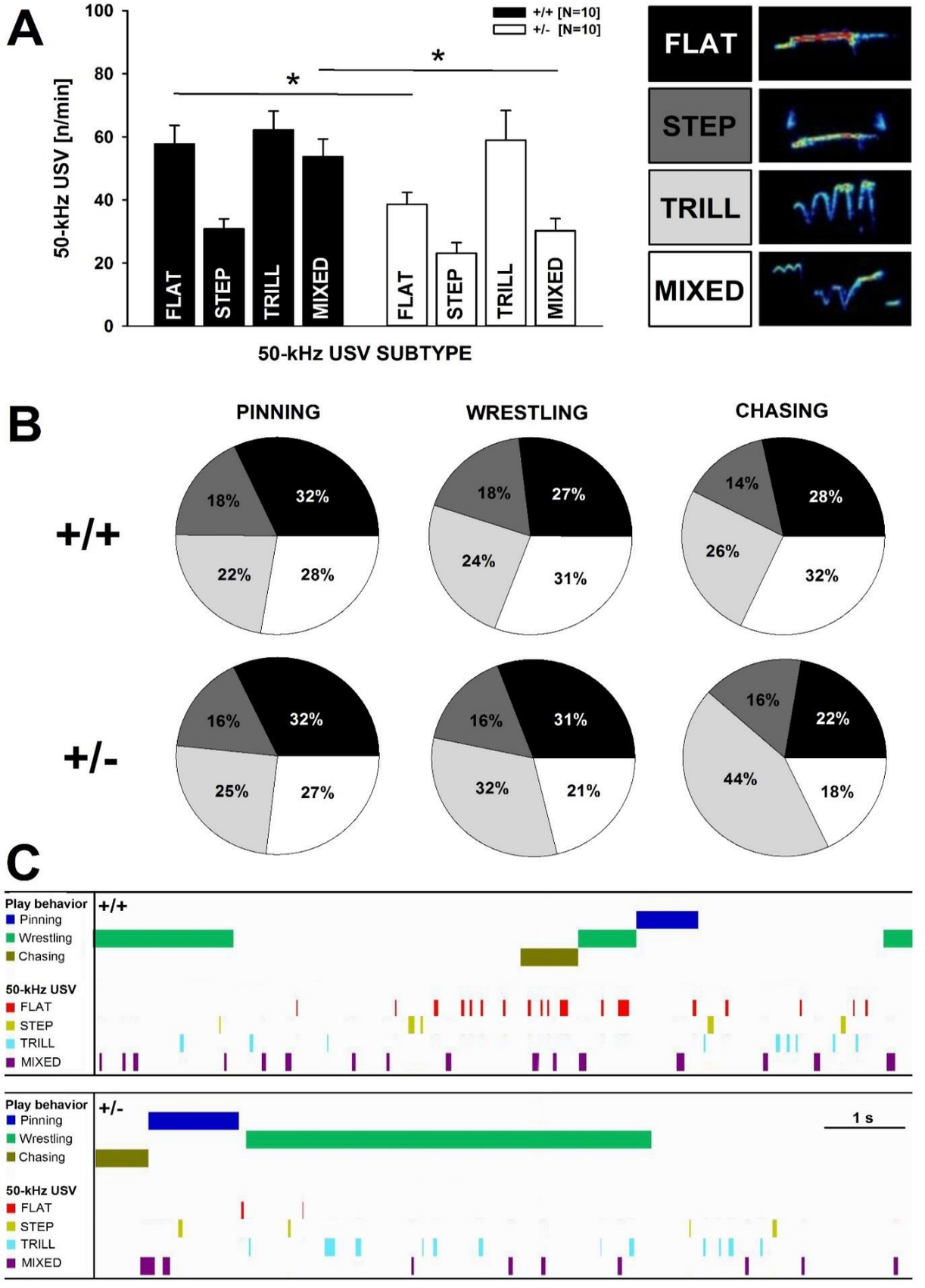

A
Time spent playing [s]
+/+ [N=10]
+/- [N=10]
Play session [n]
B
Time spent pinning [s]
C
50-kHz USV [n/min]
D
50-kHz USV [n/s]
PLAY
NO PLAY
PLAY PERIOD
E
PINNING
WRESTLING
CHASING
F
30 s
First play session
Third play session
Play behavior
Pinning
Wrestling
Chasing
Vocalization
50kHz USV

FIGURE 3


A
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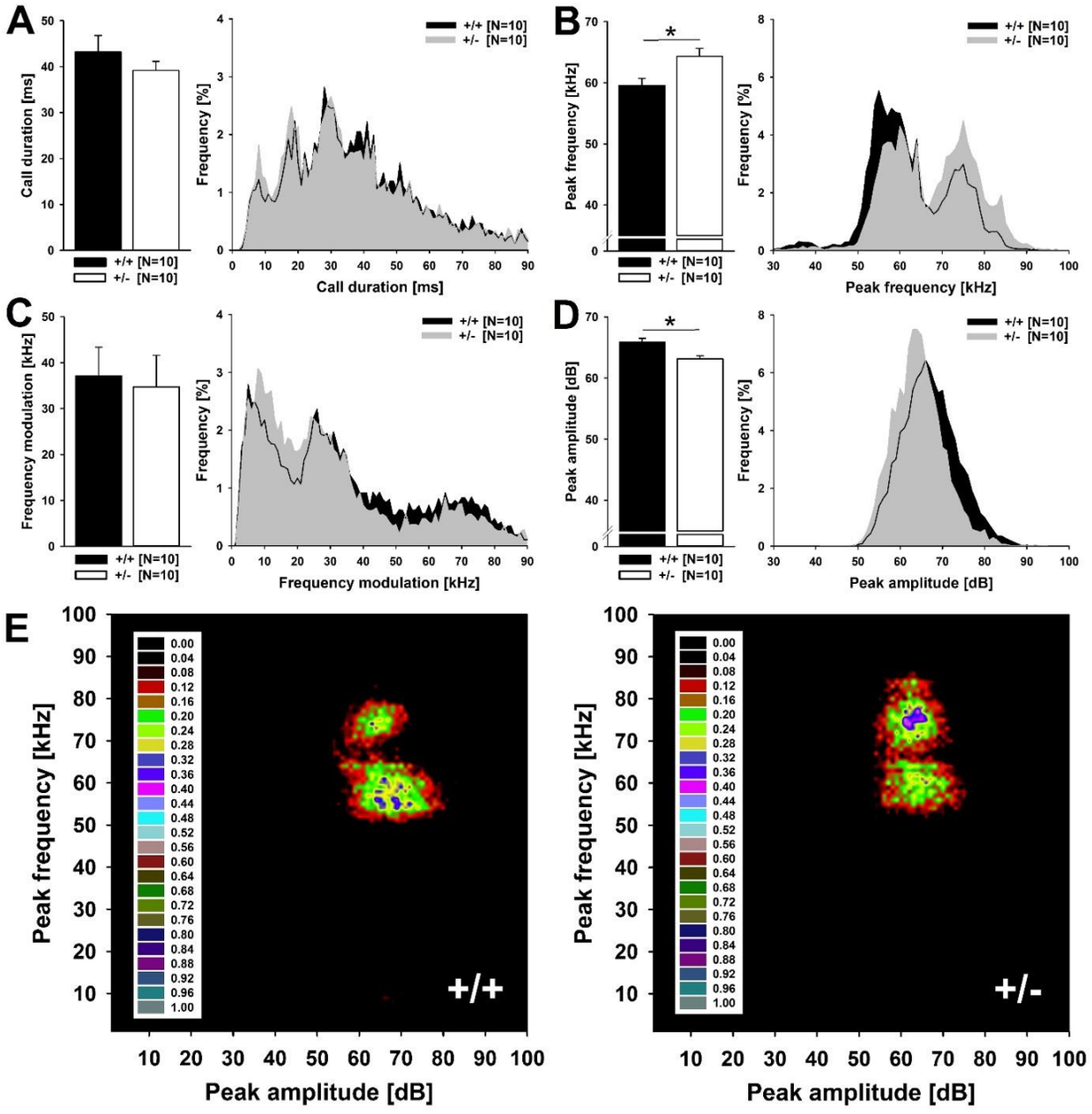
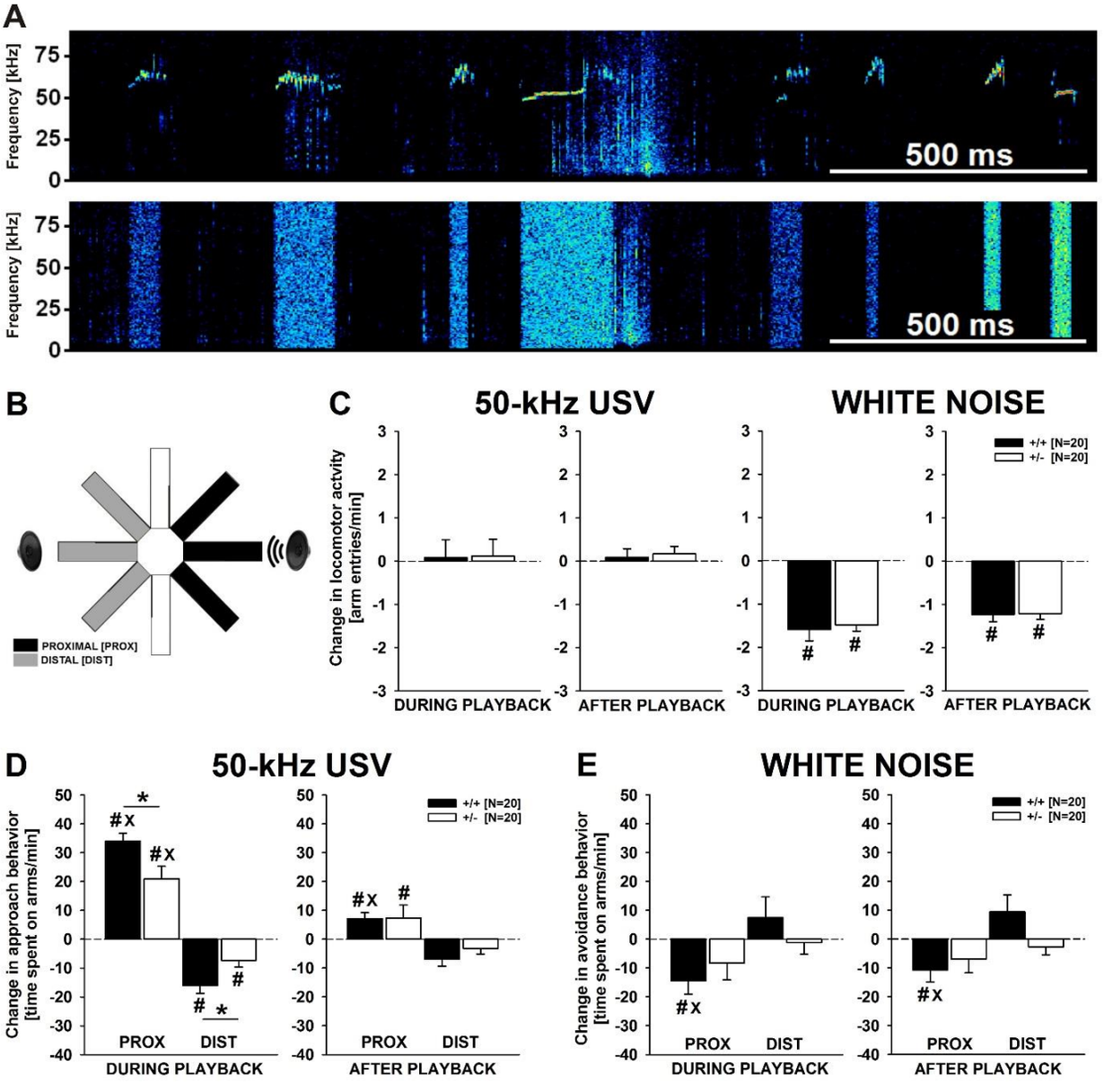
+/- [N=10]
50-kHz USV [n/min]
100
80
60
40
20
0
*
*
FLAT
STEP
TRILL
MIXED
FLAT
STEP
TRILL
MIXED
50-kHz USV SUBTYPE
FLAT
STEP
TRILL
MIXED
B
PINNING
WRESTLING
CHASING
+/+
18%
32%
22%
28%
18%
27%
24%
31%
14%
28%
26%
32%
+/-
16%
32%
25%
27%
16%
31%
32%
21%
16%
22%
44%
18%
C
Play behavior
Pinning
Wrestling
Chasing
50-kHz USV
FLAT
STEP
TRILL
MIXED
+/+
Play behavior
Pinning
Wrestling
Chasing
50-kHz USV
FLAT
STEP
TRILL
MIXED
+/-
1 s

FIGURE 4


A
Call duration [ms]
+/+ [N=10]
+/- [N=10]
Frequency [%]
Call duration [ms]
B
Peak frequency [kHz]
*
Peak frequency [kHz]
C
Frequency modulation [kHz]
Frequency modulation [kHz]
D
Peak amplitude [dB]
Peak amplitude [dB]
E
Peak frequency [kHz]
Peak amplitude [dB]
+/+
+/-

855 **Figure 5**

856



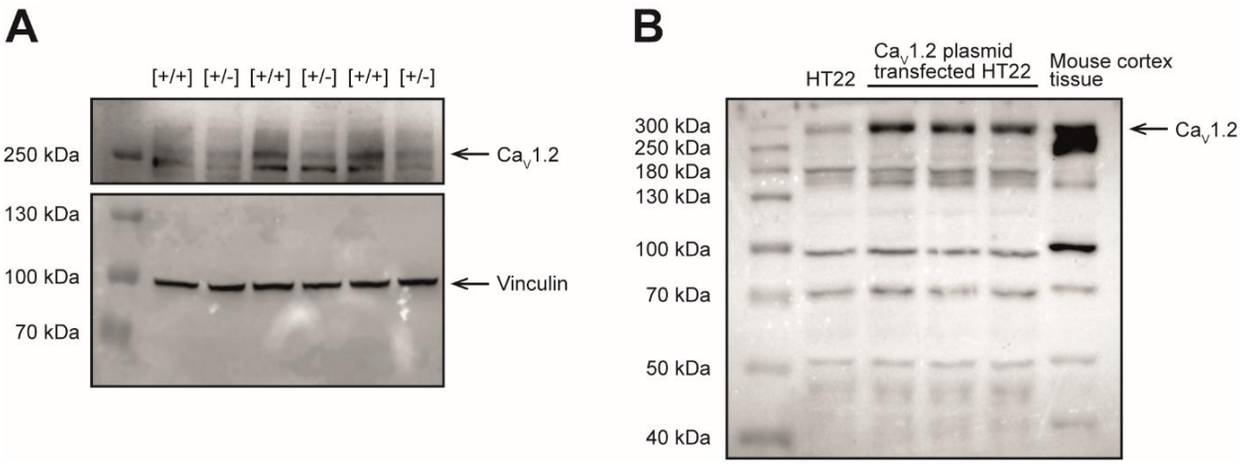
857

858

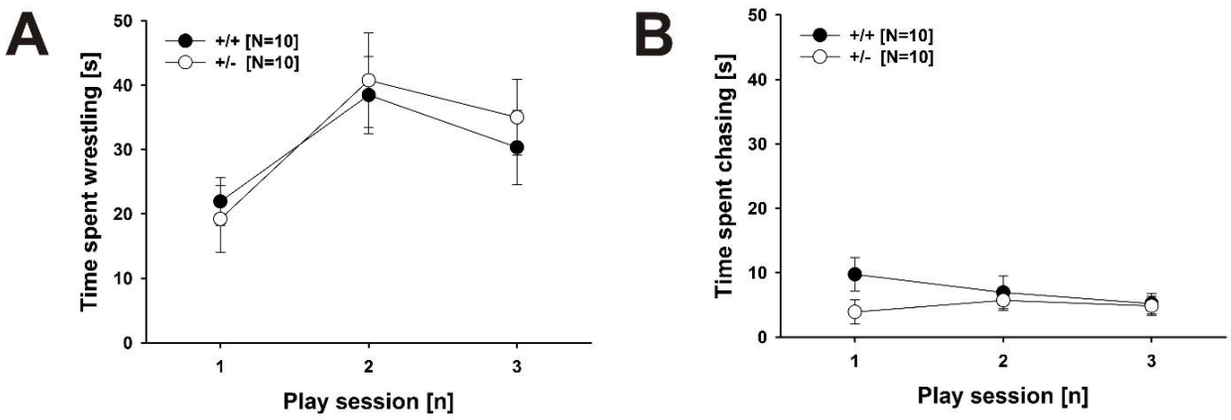
Supplementary Materials

Title: *Cacna1c* haploinsufficiency leads to pro-social 50-kHz ultrasonic communication deficits in rats

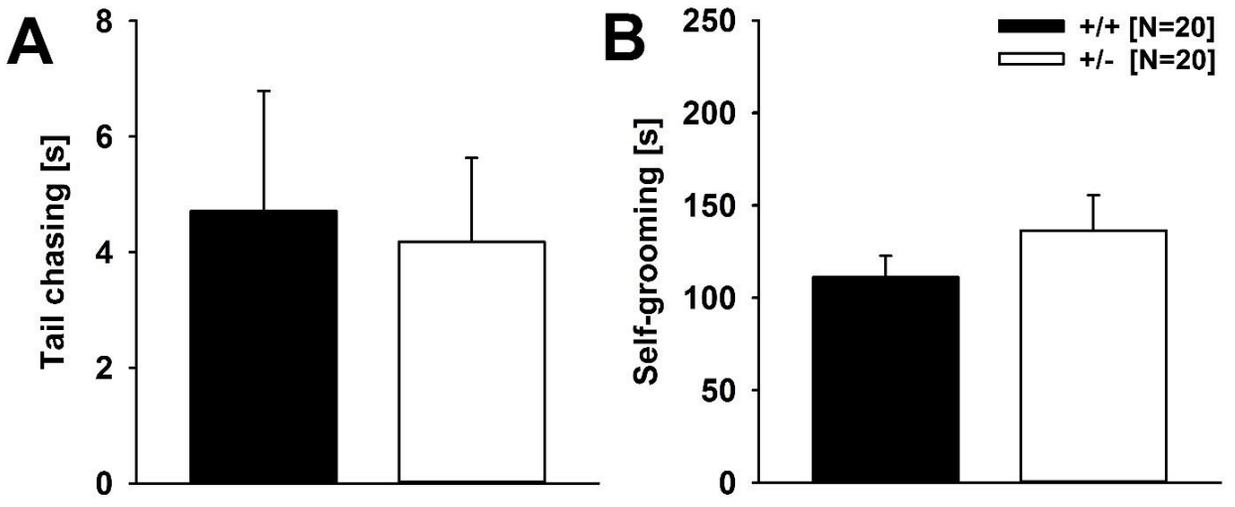
Authors: Theresa M. Kisko, Moria D. Braun, Susanne Michels, Stephanie H. Witt, Marcella Rietschel, Carsten Culmsee, Rainer K.W. Schwarting, Markus Wöhr



Supplementary Figure S1: Representative whole immunoblot from rat cortical tissue of *Cacna1c*$^{+/-}$ males ([+/-]) and *Cacna1c*$^{+/+}$ littermate controls ([+/+]). (**A**) The upper part of the membrane was incubated with anti-Cav1.2 antibody (1:500, Cat# ACC-003, Lot# ACC003AN5102, Alomone Labs, Jerusalem, Israel). The lower part of the PVDF membrane shows the expression levels of the loading control Vinculin. peqGOLD Protein-Marker V (VWR, Darmstadt, Germany) was used as marker (left lane). (**B**) The specificity of the anti-Cav1.2 antibody was validated using protein lysates from Cav1.2 plasmid transfected mouse hippocampal HT22 cells (1µg DNA, 48h). HT22 cells were a kind gift from Axel Methner and regularly tested for mycoplasma contamination (MycoAlert PLUS Mycoplasma Detection Kit, Lonza, Rockland, ME, USA). Cav1.2 was a kind gift from Diane Lipscombe (Addgene plasmid #26572). Spectra™ Multicolor High Range Protein Ladder (Thermo Fisher Scientific, Darmstadt, Germany) served as size standard (left lane).



Supplementary Figure S2: Social play behavior in *Cacna1c*$^{+/-}$ males and *Cacna1c*$^{+/+}$ littermate controls. (**A**) Time spent wrestling and (**B**) time spent chasing across the three play sessions in male *Cacna1c*$^{+/-}$ rats (white circles; N=10) and *Cacna1c*$^{+/+}$ littermate controls (black circles; N=10). Data are presented as mean±SEM.



Supplementary Figure S3: Repetitive and stereotyped patterns of behavior in *Cacna1c*$^{+/-}$ rats and *Cacna1c*$^{+/+}$ littermate controls. (**A**) Time spent tail chasing and (**B**) self-grooming in male *Cacna1c*$^{+/-}$ rats (white bars; N=20) and *Cacna1c*$^{+/+}$ littermate controls (black bars; N=20). Data are presented as mean±SEM.

Body Weight		
Behavioral Paradigm	*Cacna1c* $^{+/+}$	*Cacna1c* $^{+/-}$
50-kHz USV playback; PND 24±3	63.75±2.95 g	60.10±3.05 g
Rough-and-tumble play; PND 32-34	105.80±3.69 g	103.95±3.84 g
Repetitive behavior; PND 64±3	337.25±5.80 g	338.65±6.12 g

Notes: USV = Ultrasonic vocalizations; PND = Postnatal day

Supplementary Table S1: Body weight.

Study II

Title: Sex-dependent effects of *Cacna1c* haploinsufficiency on juvenile social play behavior and pro-social 50-kHz ultrasonic communication in rats

Authors: Theresa M. Kisko, Moria D. Braun, Susanne Michels, Stephanie H. Witt, Marcella Rietschel, Carsten Culmsee, Rainer K.W. Schwarting, Markus Wöhr

Status: in preparation for submission

Submitted to: (planned) Genes, Brains and Behavior

Sex-dependent effects of *Cacna1c* haploinsufficiency on juvenile social play behavior and pro-social 50-kHz ultrasonic communication in rats

Cacna1c and social behavior in female rats

Original Research Article

Theresa M. Kisko[1]*, Moria D. Braun[1]*, Susanne Michels[2], Stephanie H. Witt[3], Marcella Rietschel[3], Carsten Culmsee[2,4], Rainer K.W. Schwarting[1,4], Markus Wöhr[1,4]

[1]Behavioral Neuroscience, Experimental and Biological Psychology, Faculty of Psychology, Philipps-University of Marburg, Gutenbergstr. 18, D-35032 Marburg, Germany

[2]Institute of Pharmacology and Clinical Pharmacy, Philipps-University of Marburg, Karl-von-Frisch-Str. 1, D-35032 Marburg, Germany

[3]Department of Genetic Epidemiology in Psychiatry, Central Institute of Mental Health, Faculty of Medicine Mannheim, University of Heidelberg, J5, D-65189 Mannheim, Germany

[4]Center for Mind, Brain, and Behavior (CMBB), Philipps-University of Marburg, Hans-Meerwein-Str. 6, D-35032 Marburg, Germany

* shared first authorship

Correspondence should be addressed to:
Markus Wöhr, PhD
ORCID: 0000-0001-6986-5684
Behavioral Neuroscience
Experimental and Biological Psychology
Philipps-University of Marburg

Gutenbergstr. 18, 35032 Marburg, Germany
Fax: +6421 28 23610, Tel: +6421 28 23612
e-mail: markus.woehr@staff.uni-marburg.de

ABSTRACT

The cross-disorder risk gene *CACNA1C* has recently been implicated in several major neuropsychiatric disorders characterized by deficits in social behavior and communication. Recently, we found that heterozygous male *Cacna1c* rats display prominent deficits in pro-social ultrasonic communication in both the sender and receiver. The present study aimed at exploring the role of *Cacna1c* in regulating sex-specific effects in juvenile social behavior and communication, together with repetitive and stereotyped patterns of behavior, specifically after weaning during the critical period of development in juvenile rats. To this aim, we used a newly developed genetic *Cacna1c* rat model and compared wildtype (*Cacna1c*$^{+/+}$) and constitutive heterozygous (*Cacna1c*$^{+/-}$) males and females, following a truly communicative approach, including both sender and receiver. Our results show that a deletion of *Cacna1c* leads to alterations in social behavior and pro-social 50-kHz ultrasonic communication in a sex-dependent manner. Female *Cacna1c*$^{+/-}$ rats displayed increased levels of social play of social play, driven by an increase in the time spent pinning in comparison to male and female *Cacna1c*$^{+/+}$littermate controls. Irrespective of genotype, no difference in 50-kHz ultrasonic vocalization was observed in females during social play. However, similar to males *Cacna1c* deletion in females reduced behavioral responses in the minutes following playback of 50-kHz ultrasonic vocalizations. This indicates that *Cacna1c* haploinsufficiency creates alterations in social play patterns in females and has minor effects on pro-social 50-kHz ultrasonic communication in the receiver. No evidence for repetitive and stereotyped patterns of behavior was obtained. Together, these data suggest that *Cacna1c* plays a prominent role in regulating socio-affective communication and behavior in rats with relevance to sex-specific effects seen in neuropsychiatric disorders.

Key Words: $Ca_V1.2$, calcium, autism, social behavior, rough-and-tumble play, ultrasonic vocalizations, sex differences

INTRODUCTION

The cross-disorder risk gene *CACNA1C* is strongly implicated in multiple neuropsychiatric disorders characterized by social behavior and communication deficits, for example autism spectrum disorder (ASD; (Splawski *et al.*, 2004, 2005; D'Gama *et al.*, 2015; Li *et al.*, 2015)), schizophrenia (SCZ; (Green *et al.*, 2010; Nyegaard *et al.*, 2010; Schizophrenia Psychiatric Genome-Wide Association Study (GWAS) Consortium, 2011; Ripke *et al.*, 2014)), major depressive disorder (MDD; (Dao *et al.*, 2010; Green *et al.*, 2010; Rao *et al.*, 2016)), and bipolar disorder (BPD; (Ferreira *et al.*, 2008; Sklar *et al.*, 2008, 2011; Moskvina *et al.*, 2009)). *CACNA1C* codes for the α1C subunit of the voltage-gated L-type calcium channel (LTCC) $Ca_v1.2$ with voltage sensor and conduction pore, regulating depolarization-dependent calcium influx into the cell (Sinnegger-Brauns *et al.*, 2009). This is of considerable interest, particularly since LTCCs play a pivotal role in modulating neuronal excitability, synaptic plasticity, and gene expression, as experimentally demonstrated in rodent studies (Zuccotti *et al.*, 2011; Zamponi, 2016). $Ca_v1.2$ accounts for about 80 % of all LTCCs in the rodent brain (Zuccotti *et al.*, 2011; Zamponi, 2016) and thus represents a primary target for both drugs and second messengers acting on LTCCs (Zuccotti *et al.*, 2011; Zamponi, 2016). *CACNA1C* has emerged as a prime candidate susceptibility gene (Bhat *et al.*, 2012; Heyes *et al.*, 2015; Ou *et al.*, 2015), particularly because single-nucleotide polymorphisms (SNPs) within rank among the most consistent and replicable genetic findings from genome-wide association studies (GWAS) in psychiatry (Liu *et al.*, 2011; Wray *et al.*, 2012; Consortium, 2013).

Deficits in social behavior are commonly seen in all major neuropsychiatric disorders (Meyer-Lindenberg and Tost, 2012). For instance, the main characteristics of ASD include impaired social interaction and communication, together with repetitive and stereotyped patterns of behavior (Battle, 2013). Importantly, ASD-related behavioral phenotypes can be studied in rodents through a comprehensive set of behavioral assays for detecting impairments in social interaction and communication developed in the last decade (Silverman *et al.*, 2010; Wöhr and Scattoni, 2013). Traditionally, social behaviors in mouse models are assessed using the three-chambered social approach assay, with intact sociability being defined as spending more time in proximity to a conspecific over

an empty corral. Using this classic assay, Kabir et al. (2017) and Dedic et al. (2017) found that adult male forebrain *Cacna1c* null mutant mice do not show a preference for the conspecific. Lack of sociability was also seen after *Cacna1c* knockdown specifically in the prefrontal cortex (Kabir *et al.*, 2017), but not the nucleus accumbens (Terrillion, Francis, *et al.*, 2017). However, in constitutive *Cacna1c* heterozygous mice, no evidence for social deficits was obtained (Dedic *et al.*, 2017), with one study even reporting enhanced sociability in a newly developed social home cage assay (Bader *et al.*, 2011). Finally, evidence for a role of *Cacna1c* in regulating socio-affective information processing comes from a study by Jeon et al. (Jeon *et al.*, 2010), who showed that observational fear learning in mice is impaired following local $Ca_v1.2$ deletion in the anterior cingulate cortex (for a comprehensive overview on the behavioral effects of genetic modifications targeting *Cacna1c* in mice, see (Kabir, Lee and Rajadhyaksha, 2016)). Of note, however, all of the aforementioned studies investigating social impairments in *Cacna1c* mice have used adult males, thus, the role of *Cacna1c* on female social behavior has yet to be investigated. Moreover, evidence for a lack of social behavior deficits as a result of altered $Ca_v1.2$ expression levels was recently found in juvenile *Cacna1c* male rats. Nonetheless, Dao et al (2010) found that in humans *CACNA1C* is associated with sex-specific effects in females which is further paralleled by findings in *Cacna1c* heterozygous mice. Specifically, female *Cacna1c* mice with just one copy of the gene display higher levels of anxiety across numerous behavioral tests (Dao *et al.*, 2010), however, no tests for sociability were conducted.

While mice currently tend to be the most commonly used model species, rats have several advantages (Ellenbroek and Youn, 2016; Homberg, Wöhr and Alenina, 2017). Notably, rats are highly gregarious animals characterized by prominent social hierarchies (Baenninger, 1966) and a rich and complex social behavior repertoire. Importantly, rats begin interacting socially at a very young age and engage in high levels of social play behavior as juveniles, making it the most used model species to study social play (Pellis and Pellis, 2009; Siviy and Panksepp, 2011; Vanderschuren, Achterberg and Trezza, 2016). Social play behavior, also known as rough-and-tumble play, is the earliest form of mammalian social behavior that is directed at peers and not the mother. In rats, it reaches its peak during the

middle of the juvenile stage (Bolles and Woods, 1964; Panksepp, 1981; Thor and Holloway, 1984). Vital to healthy development, social play contributes more than just a high value of reward to the participants (Trezza, Baarendse and Vanderschuren, 2010). Playful social interaction during adolescence is important for adequate acquisition of social, emotional, and cognitive skills, like the expression and interpretation of communicative signals from conspecifics. Regulating and executing social play requires numerous neural systems (Vanderschuren, Achterberg and Trezza, 2016). In addition, the complex nature of the social play repertoire, including pinning, wrestling and chasing behaviors were found to be affected by selectively manipulating genetic (Homberg *et al.*, 2007), environmental (Raza *et al.*, 2015), pharmacological (Vanderschuren *et al.*, 1995) and brain mechanisms (Schneider and Koch, 2005). Of importance, Wöhr and Lukas (2015) recently reported that rats selectively bred for high levels of anxiety engaged in much less social play than rats bred for low, or non-selected anxiety levels.

Acoustic communication is an important component of the rat social behavior repertoire. Rats emit whistle-like calls in the ultrasonic range, so-called ultrasonic vocalizations (USV; (Portfors, 2007; Brudzynski, 2013; Wöhr and Schwarting, 2013)). Evidence from selective breeding, devocalization, and playback studies suggests that the various USV types serve as situation-dependent socio-affective signals and fulfill distinct communicative functions. Specifically, 22-kHz USV occur in aversive situations, such as predator exposure, social defeat, and fear learning, and are thought to reflect a negative affective state of the sender. They serve an alarm function and induce freezing behavior in the recipient (Endres, Widmann and Fendt, 2007; Wöhr and Schwarting, 2007). In contrast, 50-kHz USV are thought to reflect a positive affective state (“rat laughter”; (Panksepp, 2005)), as they occur in appetitive situations, most notably rough-and-tumble play with conspecifics. Interestingly, it was recently shown that in juvenile male *Cacna1c* rats 50-kHz USV profiles can be influenced by *Cacna1c* haploinsufficiency during rough-and-tumble play (Kisko et al, in press).

As repeatedly demonstrated in playback studies, appetitive 50-kHz USV serve important pro-social communicative functions and induce social exploratory and

approach behavior in receivers, probably by eliciting the anticipation of rewarding social contact (Wöhr and Schwarting, 2007, 2009, 2012; Seffer *et al.*, 2015; Brenes *et al.*, 2016; Engelhardt *et al.*, 2017). Like rough-and-tumble play, social approach is particularly prominent in juvenile rats (Wöhr and Schwarting, 2007), suggesting that it can be used as a behavioral readout for the incentive salience of social contact. In support for a communicative function as social contact calls acting to (re)establish or maintain social proximity, young rats were found to spend more time with conspecifics displaying higher levels of pro-social 50-kHz USV emission than those with low rates (Panksepp, Gordon and Burgdorf, 2002) and the potential for social contact also greatly increases the production of 50-kHz USV (Brudzynski and Pniak, 2002; Schwarting, Jegan and Wöhr, 2007; Wöhr *et al.*, 2008). Moreover, experimental evidence was recently provided indicating that 50-kHz USV promote and maintain playful social interactions (B. T. Himmler *et al.*, 2014). However, if rats are unable to emit 50-kHz USV, social play behavior significantly decreases (Kisko, Himmler, *et al.*, 2015), and without sufficient social play during the critical juvenile period the risk for developing severe social impairments increases. For example, it was shown that post-weaning social isolation resulted in impaired social approach to 50-kHz USV (Seffer *et al.*, 2015). Hence, social play and the concomitant emission of 50-kHz USV in the sender, together with behavioral responses to playback of 50-kHz USV in the receiver, appear to be ideal readouts for assessing behavioral deficits in social behavior and communication with relevance to neuropsychiatric disorders in rats.

Importantly, a previous study investigating the development of social behavior and communication in male juvenile *Cacna1c* rats (Kisko et al *in press*) has shown that a decrease in $Ca_v1.2$ expression levels results in impairments in ultrasonic communication in both the sender and receiver. Males with only one copy of the *Cacna1c* gene consistently emit fewer 50-kHz USV during rough-and-tumble play, display altered 50-kHz USV subtype profiles and subsequently display reduced social approach behavior in response to 50-kHz USV playback. Indicating that in juvenile male rats, *Cacna1c* may be associated with impairments in communication and incentive salience during pro-social interactions.

In the present study, our aim was to further explore the role of *Cacna1c* in regulating disorder-related behavioral phenotypes, focusing on sex-specific differences in social behavior and communication, together with repetitive and stereotyped patterns of behavior, during the critical period of development in juvenile rats. To this aim, we used a previously established genetic *Cacna1c* rat model and compared recent findings in male juvenile *Cacna1c* rats with wildtype (*Cacna1c*$^{+/+}$) and constitutive heterozygous (*Cacna1c*$^{+/-}$) females, following a truly communicative approach, including both sender and receiver. Based on our latest findings in *Cacna1c* male rats, as well as evidence from *Cacna1c* mouse studies we hypothesized that female *Cacna1c*$^{+/-}$ rats show impairments in social play behavior associated with deficient 50-kHz ultrasonic communication when compared to the *Cacna1c*$^{+/+}$ littermate controls.

MATERIALS AND METHODS

Animals and housing

Effects of *Cacna1c* haploinsufficiency were assessed in male and female constitutive heterozygous *Cacna1c*$^{+/-}$ rats (N=40) and compared to wildtype *Cacna1c*$^{+/+}$ littermate controls (N=40), with balanced representation of sexes in both groups (N=20 per genotype). Results obtained in male *Cacna1c*$^{+/-}$ and *Cacna1c*$^{+/+}$ rats were reported before (Kisko et al., in press), but extended by additional analyses where necessary for the sake of sex comparison. *Cacna1c*$^{+/-}$ rats were generated by means of zinc finger technology by SAGE Labs (now Horizon Discovery Ltd, Cambridge, UK) on a Sprague-Dawley (SD) background, following a previously established protocol (Geurts *et al.*, 2009). *Cacna1c*$^{+/-}$ rats carry a 4 base pair (bp) deletion at 460649-460652 bp in genomic sequence resulting in an early stop codon in exon 6. Homozygous *Cacna1c*$^{-/-}$ rats are embryonically lethal. Genotyping was performed as reported before (Kisko et al., in press), with the following primers being used: GCTGCTGAGCCTTTTATTGG (*Cacna1c* Cel-1 F) and CCTCCTGGATAGCTGCTGAC (*Cacna1c* Cel-1 R).

As reported before (Kisko et al., in press), a heterozygous breeding protocol was used to obtain offspring from both genotypes. To this aim, SD females (Charles River, Sulzfeld, Germany) and male *Cacna1c*$^{+/-}$ rats were paired for breeding. SD females were used because breeding efficacy is reduced in female *Cacna1c*$^{+/-}$ rats. N=8 litters with N=16.25±0.67 pups were obtained, with equal sex (t_7=0.143;

p=0.809) and genotype (t_7=0.540; p=0.606) ratios. In order to avoid litter effects, only litters with both genotypes and sexes being present were included in the experiments. Breeding was performed at the Faculty of Psychology, Philipps-University of Marburg, Germany. Approximately 2 weeks after pairing for breeding, females were individually housed and inspected daily for pregnancy and delivery. The day of birth was considered as postnatal day (PND) 0. After weaning on PND 21, rats were socially housed in groups of 4-6 with same-sex littermate partners in polycarbonate Macrolon Type IV cages (Tecniplast Deutschland GmbH, Hohenpeißenberg, Germany; 58 x 38 x 20 cm, length x width, x height) under standard laboratory conditions (22±2 °C and 40-70 % humidity) with free access to standard rodent chow and water. Rats were identified by paw tattoo, using non-toxic animal tattoo ink (Ketchum permanent tattoo inks green paste, Ketchum Manufacturing Inc., Brockville, Canada). The ink was inserted subcutaneously through a 30-gauge hypodermic needle tip into the center of the paw on PND 5±1.

Protein analysis

Protein extraction and Western blot were performed using frozen cortical tissue pieces (25-50 mg, left hemisphere) from 10-month-old female *Cacna1c*$^{+/-}$ rats (N=6) and their *Cacna1c*$^{+/+}$ littermate controls (N=6), applying a protocol described before (Kisko et al., in press). Each tissue sample was lysed in 600 µl buffer containing 50 mM Tris hydrochloride, 150 mM sodium chloride, 5 mM EDTA, 1 % (w/v) Triton X-100 and 0.5 % (w/v) sodium deoxycholate supplemented with protease and phosphatase inhibitor cocktail tablets (Roche Diagnostics, Mannheim, Germany) and homogenized with T10 basic Ultra-Turrax (IKA-Werke, Staufen, Germany) for 10 s. The homogenates were then centrifuged for 15 min at 13,000 xg and 4 °C (Heraeus Fresco^{TM} 17, Thermo Fisher Scientific, Darmstadt, Germany). The total protein amount was determined from the supernatants using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Darmstadt, Germany). Fifty µg protein per sample were loaded on a 7.5 % polyacrylamide gel. After electrophoresis, the proteins were transferred onto a PVDF membrane (Roche Diagnostics, Mannheim, Germany) and incubated with anti-Ca_v1.2 (1:500; Cat# ACC-003; Lot# ACC003AN5102; Alomone Labs, Jerusalem, Israel) and anti-Vinculin antibodies (1:20,000; Sigma-Aldrich, München, Germany) overnight at 4 °C. Protein detection was realized using peroxidase labeled secondary antibodies

(Vector Laboratories, Burlingame, CA, USA) and luminol based HRP-Juice Plus (PJK GmbH, Kleinblittersdorf, Germany). The resulting chemiluminescence was imaged with a ChemiDoc XRS system (Bio-Rad Laboratories, Hercules, CA, USA). Protein quantification was performed using Bio-Rad Image Lab™ Software. Unless otherwise stated, all reagents were purchased from Sigma-Aldrich (München, Germany).

Behavioral phenotyping

Behavioral phenotypes were assessed in male and female *Cacna1c*$^{+/-}$ rats and compared to *Cacna1c*$^{+/+}$ littermate controls by means of our established 50-kHz USV radial maze playback paradigm (PND 24±3), rough-and-tumble play behavior and pro-social 50-kHz USV (PND 32-34), as well as repetitive and stereotyped patterns of behavior (PND 64±3). In addition, olfactory habituation and dishabituation was performed (PND 75±3). All rats were tested in all four behavioral assays. Behavioral experiments were carried out during the light phase of a 12:12 h light / dark cycle (lights on at 06:00 h). Rats were handled for three consecutive days prior to behavioral testing in a standardized way for 5 min. Behavioral analysis was performed by an experienced observer blind to experimental condition.

Rough-and-tumble play and pro-social 50-kHz ultrasonic vocalizations

On PND 32-34, rough-and-tumble play behavior and the emission of pro-social 50-kHz USV were measured, as reported before (Kisko et al., in press) and using a modified protocol previously established (Lukas and Wöhr, 2015). In rats, rough-and-tumble play behavior peaks around the age of PND 30-40 (Bolles and Woods, 1964; Panksepp, 1981; Thor and Holloway, 1984). On three consecutive days, pairs of juvenile rats were allowed to socially interact for 5 min (referred to as play phase) in an, at first, unfamiliar observation arena (35 x 35 cm, with Plexiglas walls; floor covered with 1 cm of fresh bedding) after one rat of the pair being habituated to the test environment for 2 min (referred to as anticipation phase). A three days protocol was applied in order to assess changes in rough-and-tumble play and 50-kHz USV emission induced by play experience, such as anticipatory 50-kHz USV (Knutson et al., 1998). Rats were always paired with a same-sex, same-genotype, age-matched non-littermate and non-cagemate partner, since it is not yet possible to identify the sender of pro-social 50-kHz USV during rough-and-tumble play

behavior in a reliable manner. To enhance the level of social motivation, subject rats were socially isolated for 24 h prior testing in a Makrolon type III cage (265 x 150 x 425 mm, plus high stainless-steel covers; Tecniplast Deutschland GmbH), and isolation was maintained throughout the three days testing period. For behavioral analyses, a digital camera (TK-1281 Color Video Camera, JVC, Yokohama, Japan) was used and connected to an external multimedia hard drive (ScreenPlay Pro HD, Iomega, San Diego, CA, USA). The following behavioral measures were scored by an experienced observer using The Observer XT (Noldus, Wagenigen, The Netherlands): duration of rough-and-tumble play (including pinning, wrestling, and chasing), duration of social investigation (including sniffing the anogenital and head/neck regions of the partner), and duration of physical contact, with the latter two being considered as social but non-playful behaviors. Pinning was defined as one rat lying with its dorsal surface on the floor with the other rat standing over it. Wrestling was scored when a group of play-specific behaviors, including wrestling, boxing, and pouncing, occurred. Chasing was defined as moving in the direction of or pursuing the partner while the partner is moving away. Pro-social 50-kHz USV were recorded using an UltraSoundGate Condenser Microphone (CM16; Avisoft Bioacoustics, Berlin, Germany) placed 35 cm above the floor of the center of the observation arena. Rough-and-tumble play behavior and the emission of pro-social 50-kHz USV were measured under red light (~28 lux).

Playback of pro-social 50-kHz ultrasonic vocalizations

On PND 24±3, social exploratory and approach behavior in response to pro-social 50-kHz USV was assessed on an elevated radial eight-arm maze (arms: 40.5 x 9.8 cm) under red light (~10 lux) , as reported before (Kisko et al., in press) and according to a modified playback protocol previously established (Wöhr and Schwarting, 2007). Acoustic stimuli were presented through an ultrasonic loudspeaker (ScanSpeak, Avisoft Bioacoustics) placed 20 cm away from the end of one arm. An additional, but inactive loudspeaker was arranged symmetrically at the opposite arm as a visual control. Two acoustic stimuli were used: (I) pro-social 50-kHz USV and (II) White Noise; the latter serving as a time- and amplitude-matched acoustic stimulus control (Seffer, Schwarting and Wöhr, 2014). After an initial 15 min habituation period, each subject rat was exposed to 1 min playback

presentations of 50-kHz USV and White Noise, separated by a 10 min inter-stimulus interval. Stimulus order was counterbalanced to account for possible sequence effects. The session ended after an additional 10 min post-stimulus phase. Behavior was monitored by a video camera (Panasonic WV-BP 330/GE, Hamburg, Germany) mounted centrally above the arena. Total number of arm entries served as a measure for locomotor activity. Change values were calculated by subtracting the total number of arm entries per minute during the 5 minutes baseline period before playback from the total number of arm entries per minute during and after 50-kHz USV and White Noise playback, respectively. Number of arm entries proximal and distal to the active ultrasonic loudspeaker and time spent thereon were used to quantify approach and avoidance behavior, respectively. Change values were calculated by subtracting the time spent on proximal and distal arms per minute during the 5 minutes baseline period before playback from the time spent on proximal and distal arms per minute during and after 50-kHz USV playback. USV were monitored with two ultrasonic condenser microphones (CM16, Avisoft Bioacoustics) placed next to the loudspeakers.

Recording and analysis of ultrasonic vocalizations

UltraSoundGate Condenser CM16 Microphones (Avisoft Bioacoustics) sensitive to frequencies of 15–180 kHz (flat frequency response between 25 and 140 kHz; ±6 dB) were used for USV recordings. They were connected via an UltraSoundGate 416H USB audio device (Avisoft Bioacoustics) to a personal computer, where acoustic data were recorded with a sampling rate of 250,000 Hz in 16-bit format (recording range: 0-125 kHz) by Avisoft RECORDER USGH. For acoustical analysis, recordings were transferred to Avisoft SASLab Pro (version 4.50). High resolution spectrograms (frequency resolution: 488 Hz; time resolution: 0.512 ms) were obtained through a fast Fourier transformation (512 FFT length, 100 % frame, Hamming window and 75 %-time window overlap). Call detection of pro-social 50-kHz USV emitted by juvenile rats during rough-and-tumble play was provided by an experienced observer, who manually counted the numbers of USV in 20 s time bins. If two 50-kHz USV elements were at least 10 ms apart, two independent 50-kHz USV were counted (Wöhr *et al.*, 2015). Of note, aversive 22-kHz USV occurred very rarely and were therefore not included in the analysis. During playback of pro-social 50-kHz USV, 22-kHz and 50-kHz USV occurred. USV emitted within a

frequency range of 20-33 kHz were considered as 22-kHz USV and USV with peak frequencies higher than 33 kHz as 50-kHz USV (Engelhardt *et al.*, 2017).

Repetitive and stereotyped patterns of behavior

On PND 64±3, repetitive and stereotyped patterns of behavior were tested in a clean Makrolon type III cage (265 x 150 x 425 mm, plus high stainless-steel covers; Tecniplast Deutschland GmbH) without bedding material, as reported before (Kisko et al., in press). For behavioral analyses, a digital camera (TK-1281 Color Video Camera, JVC) was used and connected to an external multimedia hard drive (ScreenPlay Pro HD, Iomega). Repetitive and stereotyped patterns of behavior were assessed by measuring the duration of self-grooming and circling behavior during tail-chasing. For assessing locomotor activity, the test cage was virtually divided in two halves by a line and the numbers of line crossings and rearing events were counted. Testing was performed under white light (~30 lux) conditions for 20 min.

Olfactory habituation and dishabituation

On PND 75±3, olfactory habituation and dishabituation was tested in a clean Makrolon type III cage (265 x 150 x 425 mm, plus high stainless-steel covers; Tecniplast Deutschland GmbH) with fresh bedding material. A cage with fresh bedding was used for each subject rat to avoid odor contamination. Odor-saturated cotton-tipped wooden applicators (wooden cotton swabs, sterile; length 150 mm, diameter of tip 4-5.5 mm; Rotilabo, Karlsruhe, Germany) were used to deliver odor stimuli. To reduce novelty-induced exploratory activities, rats were first habituated to testing enclosure and procedure by exposing each subject rat for 45 min to the cage, with a clean cotton-tipped wooden applicator suspended from the cage lid to be well within reach of the subject rat. During testing, each subject rat was presented with five different odors, i.e. plain tap water, two non-social odors, and two social odors, as described previously (Yang and Crawley, 2009). The test consisted of 15 sequential 2 min trials, with three consecutive trials per odor and an inter-trial interval of 1 min: three presentations of plain tap water, three presentations of banana odor (Banana Cream Flavor, 3.7 ml flask, diluted 1:100 with tap water; LorAnn Oils, Lansing, MI, USA), three presentations of almond odor (Almond Flavor, 3.7 ml flask, diluted 1:100 with tap water; LorAnn Oils), three

presentations of social odor from social cage 1, and three presentations of social odor from social cage 2. Non-social and social odor presentations were counterbalanced within the same odor category. Water, banana odor, and almond odor stimuli were prepared by dipping the cotton tip briefly into the solution. Social odors were obtained from home cages of two unfamiliar same-sex litters by wiping the cotton-tipped wooden applicator across the bottom of the relevant soiled cage in a zig-zag motion. All odors were stored and kept away from the testing room; tap water and the two non-social odors were stored in tightly sealed plastic vials; social odors were kept on a cart outside of the testing room. For behavioral analyses, a digital camera (TK-1281 Color Video Camera, JVC) was used and connected to an external multimedia hard drive (ScreenPlay Pro HD, Iomega). Assessment of olfactory habituation and dishabituation was done by a well-trained observer measuring the time spent sniffing the odor using stopwatches. A subject rat was considered to be sniffing the odor when its nose was within the radius of 2 cm around the cotton-tipped wooden applicator. In the rare case of the cotton-tipped wooden applicator being pulled down by the subject rat, scoring of the time spent sniffing was stopped and restarted the next trial. One subject rat was excluded from statistical analysis due to data loss. Olfactory habituation and dishabituation was tested once under white light (~30 lux) conditions.

Statistical Analysis

For comparing rough-and-tumble play behavior and pro-social 50-kHz USV between genotypes, analysis of variances (ANOVAs) for repeated measurements were calculated with the between-subject factor genotype (G) and the within-subject factor day (D; interactions: DxG). Playback of pro-social 50-kHz USV was analyzed using ANOVAs for repeated measurements with the between-subject factor genotype (G) and the within-subject factors time (T) and preference (P; interactions: TxG, PxG, TxP, TxPxG). ANOVAs were followed by paired and unpaired t-tests when appropriate. Repetitive and stereotyped patterns of behavior, line crossings, and rearing events were compared between genotypes by means of unpaired t-tests. *Cacna1c*$^{+/+}$ males served as reference, with *Cacna1c*$^{+/+}$ and *Cacna1c*$^{+/-}$ females being independently compared to *Cacna1c*$^{+/+}$ males using ANOVAs or unpaired t-tests. Olfactory habituation and dishabituation was compared using an ANOVA with the between-subject factors genotype (G)

and sex (S) and the within-subject factor stimulus exposure (E; interactions: ExG, ExS, ExSxG). Cav1.2 protein levels were compared using unpaired t-tests. A p-value of <0.050 was considered statistically significant.

RESULTS

In the present study, we explored the role of *Cacna1c* in regulating behavioral phenotypes, focusing on socio-affective communication after weaning during the critical developmental period of adolescence in female rats. To this aim, we used a newly developed genetic *Cacna1c* rat model and applied a truly reciprocal approach for studying communication through pro-social 50-kHz USV, including both sender and receiver. Effects of *Cacna1c* haploinsufficiency were assessed in female constitutive heterozygous *Cacna1c*$^{+/-}$ rats (N=20) and compared to wildtype *Cacna1c*$^{+/+}$ littermate controls (N=20), following an experimental approach recently applied in male rats (Kisko et al., in press). As shown by western blot using cortical tissue, $Ca_V1.2$ protein levels of *Cacna1c*$^{+/-}$ females are reduced by slightly more than 50 % in the brain, as compared to *Cacna1c*$^{+/+}$ littermate controls (t_{10}=3.942; p=0.003; Figure 1), in line with findings obtained in male rats (Kisko et al., in press).

Body weight

Body weight differed between genotypes in females (Table 1). Most prominent genotype differences were seen on PND 24±3 when playback of pro-social 50-kHz USV was conducted, with *Cacna1c*$^{+/-}$ females weighing about 20 % less than *Cacna1c*$^{+/+}$ littermate controls (t_{37}=4.245; p<0.001). A week later, on PND 32-34, body weight differences were still evident during rough-and-tumble play behavior and pro-social 50-kHz USV recordings, with *Cacna1c*$^{+/-}$ females weighing now only about 10 % less than *Cacna1c*$^{+/+}$ littermate controls (t_{18}=3.039; p=0.007). Another four weeks later then, on PND 64±3, during the assessment of repetitive and stereotyped patterns of behavior, however, no genotype differences were evident anymore (t_{38}=1.059; p=0.296). In males, in contrast, there were no genotype differences in body weight, as previously reported (Kisko et al., in press).

Rough-and-tumble play behavior

Social play behavior differed between genotypes in females. *Cacna1c*$^{+/-}$ females spent more time playing than *Cacna1c*$^{+/+}$ littermate controls (G: $F_{1,18}$=5.293;

p=0.034; Figure 2A) and tended to display more play events (G: $F_{1,18}$=4.049; p=0.059). Increased social play behavior in *Cacna1c*$^{+/-}$ females was due to elevated levels of pinning behavior, as reflected in a higher pin duration (G: $F_{1,18}$=5.468; p=0.031; Figure 2B). Furthermore, time spent wrestling tended to be enhanced in *Cacna1c*$^{+/-}$ females (G: $F_{1,18}$=3.131; p=0.094; Suppl. Figure S1A), while chasing was not affected by genotype (G: $F_{1,18}$=2.343; p=0.143; Suppl. Figure S1B). When performing more detailed genotype comparisons across test days, the time engaging in social play behavior did not differ between genotypes on the first day (t_{18}=1.178; p=0.254). However, after the initial play session prominent genotype differences were evident, with *Cacna1c*$^{+/-}$ females spending more time playing than *Cacna1c*$^{+/+}$ littermate controls on the second and third day (t_{18}=2.239; p=0.038 and t_{18}=2.517; p=0.022; respectively). Genotype differences on the second and third day were driven by increases in time spent pinning (t_{18}=2.616; p=0.017 and t_{18}=2.435; p=0.026; respectively), while pinning did not differ on the first day (t_{18}=0.766; p=0.454). Wrestling behavior had a relatively minor impact on the overall genotype differences in social play behavior across the three test days (t_{18}=0.966; p=0.347; t_{18}=1.504; p=0.150 and t_{18}=1.773; p=0.093; respectively).

When comparing social play behavior between test days, the time engaged in playful social interactions and numbers of play events increased, irrespective of genotype (D: $F_{2,36}$=27.218; p<0.001; DxG: $F_{2,36}$=1.879; p=0.167 and D: $F_{2,36}$=13.164; p<0.001; DxG: $F_{2,36}$=1.173; p=0.321; respectively). Both *Cacna1c*$^{+/-}$ females and *Cacna1c*$^{+/+}$ littermate controls spent more time playing on the third than the first day (t_9=5.902; p<0.001 and t_9=3.258; p=0.010; respectively). Regardless of genotype, this was driven by an increase in the amount of time spent wrestling (D: $F_{2,36}$=9.530; p<0.001; DxG: $F_{2,36}$=0.409; p=0.667). Moreover, the time engaged in pinning showed an increase, with both genotypes spending more time pinning over each test day (D: $F_{2,36}$=44.324; p<0.001). However, the increase in pinning was most prominent in *Cacna1c*$^{+/-}$ females (DxG: $F_{2,36}$=4.282; p=0.021). While *Cacna1c*$^{+/+}$ littermate controls displayed a comparatively moderate increase in the duration of pinning from the first to the third test day (t_9=3.736; p=0.005), a particularly strong increase was evident in *Cacna1c*$^{+/-}$ females (t_9=9.502; p<0.001). Chasing decreased, irrespective of genotype (D: $F_{2,36}$=3.480; p=0.042; DxG: $F_{2,36}$=1.723; p=0.193).

Duration and numbers of non-play social behaviors were not affected by genotype (G: $F_{1,18}$=1.488; p=0.238 and $F_{1,18}$=0.493; p=0.491; respectively), with individual aspects, such as sniffing and physical contact, not differing between genotypes (G: $F_{1,18}$=2.654; p=0.121 and $F_{1,18}$=0.052; p=0.822; respectively; Suppl. Figure S1C&D). When comparing non-play social behaviors between test days, regardless of genotype, there was a decrease in the duration and numbers of social interactions (D: $F_{2,36}$=24.648; p<0.001; DxG: $F_{2,36}$=0.782; p=0.465 and D: $F_{2,36}$=7.782; p=0.002; DxG: $F_{2,36}$=0.759; p=0.475; respectively). Specifically, the time spent sniffing and engaging in physical contact decreased over testing days, irrespective of genotype (D: $F_{2,36}$=22.765; p<0.001; DxG: $F_{2,36}$=0.318; p=0.729; and D: $F_{2,36}$=3.629; p=0.037; DxG: $F_{2,36}$=2.250; p=0.120; respectively).

In males, there were no genotype differences in social play behavior, as previously reported (Kisko et al., in press). When comparing female and male *Cacna1c*$^{+/+}$ littermate controls, no differences in time spent playing and number of play events were seen (S: $F_{1,18}$=0.767; p=0.393 and $F_{1,18}$=0.380; p=0.545; respectively), with the individual play components, pinning, wrestling, and chasing, not differing between sexes (S: $F_{1,18}$=0.014; p=0.907; $F_{1,18}$=1.158; p=0.296; and $F_{1,18}$=3.042; p=0.098; respectively). However, when comparing *Cacna1c*$^{+/-}$ females to male *Cacna1c*$^{+/+}$ littermate controls, elevated levels of pinning behavior (S: $F_{1,18}$=5.420; p=0.032), but not wrestling and chasing behavior (S: $F_{1,18}$=0.320; p=0.579 and $F_{1,18}$=0.446; p=0.513; respectively), were seen, despite similar levels of time spent playing and number of play events (S: $F_{1,18}$=2.049; p=0.169 and G: $F_{1,18}$=2.124; p=0.162; respectively). While *Cacna1c*$^{+/-}$ females displayed similar levels of pinning behavior during the first play session (t_{18}=0.968; p=0.346), *Cacna1c*$^{+/-}$ females displayed more pinning behavior than male *Cacna1c*$^{+/+}$ littermate controls starting from the second play session (t_{18}=2.430; p=0.026 and t_{18}=2.228; p=0.039; respectively).

Pro-social 50-kHz ultrasonic vocalizations during rough-and-tumble play

During rough-and-tumble play, emission of 50-kHz USV in females did not differ between genotypes (G: $F_{1,18}$=1.100; p=0.308; Figure 2C; representative ethograms are shown in Figure 2D). Moreover, there was no difference between genotypes in emission of 50-kHz USV during the anticipation phase (G: $F_{1,18}$=0.039; p=0.845).

When comparing 50-kHz USV emission across testing days, regardless of genotype, there was an increase in 50-kHz USV emitted during the anticipation phase (D: $F_{2,36}$=7.570; p=0.002; DxG: $F_{2,36}$=1.975; p=0.154) as well as during playful social interactions (D: $F_{2,36}$=34.872; p<0.001; DxG: $F_{2,36}$=2.402; p=0.105). *Cacna1c*$^{+/-}$ females and *Cacna1c*$^{+/+}$ littermate controls both increased 50-kHz USV emission in anticipation of playful social interactions from the first to the third test day (t_9=2.569; p=0.030 and t_9=2.491; p=0.034; respectively). Likewise, *Cacna1c*$^{+/-}$ females and *Cacna1c*$^{+/+}$ littermate controls both increased 50-kHz USV during playful social interactions between test days (t_9=14.219; p<0.001 and t_9=3.638; p=0.005; respectively).

In contrast to females, *Cacna1c*$^{+/-}$ males emitted fewer 50-kHz USV than *Cacna1c*$^{+/+}$ littermate controls while engaged in playful social interactions, as previously reported (Kisko et al., in press). When comparing female and male *Cacna1c*$^{+/+}$ littermate controls, no difference in 50-kHz USV emitted during the anticipation phase was seen (S: $F_{1,17}$=2.354; p=0.143), yet during playful social interactions female *Cacna1c*$^{+/+}$ littermate controls vocalized less than male *Cacna1c*$^{+/+}$ littermate controls (S: $F_{1,18}$=7.446; p=0.014). This sex difference was clearly evident during the first and third play session (t_{17}=2.864; p=0.011 and t_{18}=2.129; p=0.047; respectively), with a trend for the second play session (t_{18}=1.802; p=0.088). A similar pattern was obtained when comparing *Cacna1c*$^{+/-}$ females to male *Cacna1c*$^{+/+}$ littermate controls. Specifically, while no difference in 50-kHz USV emitted during the anticipation phase was seen (S: $F_{1,17}$=3.436; p=0.081), female *Cacna1c*$^{+/-}$ females vocalized less than male *Cacna1c*$^{+/+}$ littermate controls during playful social interactions (S: $F_{1,18}$=13.114; p=0.002), with prominent differences being evident during all three play sessions (t_{17}=4.066; p=0.001; t_{18}=3.212; p=0.005 and t_{18}=2.146; p=0.046; respectively).

Behavioral changes evoked by pro-social 50-kHz ultrasonic vocalizations

Playback of pro-social 50-kHz USV but not the acoustic control stimulus White Noise (Figure 3A) induced social exploratory behavior in females, as demonstrated by means of our established 50-kHz USV radial maze playback paradigm (Figure 5B). Specifically, social exploratory behavior induced by playback of pro-social 50-kHz USV was reflected in an increase in total arm entries during as compared to baseline before playback, irrespective of genotype (T: $F_{1,38}$=10.826; p=0.002; TxG:

$F_{1,38}$<0.001; p=0.987; Figure 3C, left). Both *Cacna1c*$^{+/-}$ females and *Cacna1c*$^{+/+}$ littermate controls displayed more total arm entries during playback than before (T: $F_{1,19}$=7.724; p=0.006 and $F_{1,19}$=4.148; p=0.028; one-tailed; respectively). No social exploratory behavior was seen after 50-kHz USV playback (T: $F_{1,38}$=0.183; p=0.671; TxG: $F_{1,38}$=0.412; p=0.525). Importantly, increased social exploratory behavior was specifically seen in response to playback of pro-social 50-kHz USV, with the acoustic stimulus control, White Noise, inducing behavioral inhibition (T: $F_{1,38}$=136.942; p<0.001; TxG: $F_{1,38}$=0.325; p=0.572; Figure 3C, right) and arm avoidance (T: $F_{1,38}$=9.489; p=0.004; TxG: $F_{1,38}$=0.447; p=0.508; P: $F_{1,38}$=12.527; p=0.001; PxG: $F_{1,38}$=0.077; p=0.783; TxP: $F_{1,38}$=0.477; p=0.494; TxPxG: $F_{1,38}$=0.016; p=0.901; Figure 3E, left). Both *Cacna1c*$^{+/-}$ females and *Cacna1c*$^{+/+}$ littermate controls displayed reduced total arm entries during playback of White Noise than before (T: $F_{1,19}$=141.061; p<0.001 and $F_{1,19}$=42.263; p<0.001; respectively). Behavioral inhibition induced by White Noise was long-lasting and still evident after playback (T: $F_{1,38}$=124.241; p<0.001; TxG: $F_{1,38}$=3.076; p=0.087), again associated with arm avoidance (T: $F_{1,38}$=4.943; p=0.032; TxG: $F_{1,38}$=0.288; p=0.595; P: $F_{1,38}$=18.116; p<0.001; PxG: $F_{1,38}$=0.167; p=0.685; TxP: $F_{1,38}$=0.078; p=0.781; TxPxG: $F_{1,38}$=0.123; p=0.728; Figure 3E, right). Both genotypes continued to display reduced total arm entries after playback as compared to baseline (T: $F_{1,19}$=112.034; p<0.001 and $F_{1,19}$=35.082; p<0.001; respectively).

Enhanced social exploratory activity in response to playback of pro-social 50-kHz USV was mainly driven by approach behavior towards the sound source, i.e. the active ultrasonic loudspeaker. This was reflected by a strong preference for proximal arms, resulting from a marked increase in the time spent on proximal arms and a decrease in the time spent on distal arms (T: $F_{1,38}$=47.640; p<0.001; TxG: $F_{1,38}$=9.675; p=0.004; P: $F_{1,38}$=105.403; p<0.001; PxG: $F_{1,38}$=0.243; p=0.625; TxP: $F_{1,38}$=55.572; p<0.001; TxPxG: $F_{1,38}$=0.054; p=0.818; Figure 3D, left). Both *Cacna1c*$^{+/-}$ females and *Cacna1c*$^{+/+}$ littermate controls displayed social approach behavior and spent more time proximal during playback than before (T: $F_{1,19}$=23.980; p<0.001 and $F_{1,19}$=55.791; p<0.001; respectively), but less time distal (T: $F_{1,19}$=13.065; p=0.002 and $F_{1,19}$=5.535; p=0.030; respectively). This led to a preference for proximal over distal arms in both genotypes (P: $F_{1,19}$=22.139; p<0.001 and $F_{1,19}$=39.184; p<0.001; respectively). Importantly, more pronounced genotype effects were seen in the minutes following 50-kHz USV playback (T:

603 $F_{1,38}$=0.965; p=0.332; TxG: $F_{1,38}$=7.625; p=0.009; P: $F_{1,38}$=20.035; p<0.001; PxG:
604 $F_{1,38}$=0.413; p=0.524; TxP: $F_{1,38}$=5.406; p=0.026; TxPxG: $F_{1,38}$=0.016; p=0.901;
605 Figure 3D, right). While *Cacna1c*$^{+/+}$ littermate controls continued displaying a
606 preference for proximal over distal arms (P: $F_{1,19}$=6.773; p=0.017), no clear
607 preference was evident in *Cacna1c*$^{+/-}$ females (P: $F_{1,19}$=1.555; p=0.228). This is
608 due to the fact that *Cacna1c*$^{+/+}$ littermate controls but not *Cacna1c*$^{+/-}$ females kept
609 spending more time proximal after playback than before (T: $F_{1,19}$=8.057; p=0.011
610 and $F_{1,19}$=0.406; p=0.531; respectively). Irrespective of genotype, time spent distal
611 did not differ from baseline (T: $F_{1,19}$=0.220; p=0.644 and $F_{1,19}$=3.311; p=0.085;
612 respectively).
613 In contrast to females, social approach behavior in response to playback of pro-
614 social 50-kHz USV was strongly dependent on genotypes in males, with *Cacna1c*$^{+/-}$
615 males displaying lower levels of social approach behavior than *Cacna1c*$^{+/+}$
616 littermate controls, as previously reported (Kisko et al., in press). When comparing
617 female and male *Cacna1c*$^{+/+}$ littermate controls, social exploratory behavior
618 induced by playback of pro-social 50-kHz USV did not differ, i.e. in the increase in
619 the total number of arm entries (t_{38}=1.417; p=0.165), and there was no difference
620 in social approach behavior, i.e. in the increase in the time spent proximal
621 (t_{38}=1.031; p=0.309). Behavioral inhibition induced by playback of White Noise did
622 also not differ (t_{38}=0.978; p=0.334). However, when comparing *Cacna1c*$^{+/-}$ females
623 to male *Cacna1c*$^{+/+}$ littermate controls, exploratory behavior induced by playback
624 of pro-social 50-kHz USV did not differ (t_{38}=1.664; p=0.104), yet social approach
625 behavior was lower in *Cacna1c*$^{+/-}$ females than in male *Cacna1c*$^{+/+}$ littermate
626 controls (t_{38}=2.069; p=0.045). Behavioral inhibition induced by playback of White
627 Noise did not differ (t_{38}=0.660; p=0.513).
628
629 **Response calls evoked by pro-social 50-kHz ultrasonic vocalizations**
630 Some female receiver rats started to emit USV in response to 50-kHz USV
631 playback, while no USV were detected during White Noise exposure. Both
632 *Cacna1c*$^{+/-}$ females and *Cacna1c*$^{+/+}$ littermate controls emitted more 50-kHz USV
633 during 50-kHz USV playback than before (t_{19}=2.668; p=0.015 and t_{19}=3.322;
634 p=0.004; respectively), although 50-kHz USV occurred only very rarely with less
635 than one call per minute. In contrast, a substantial amount of 22-kHz USV was
636 emitted in response to playback of 50-kHz USV (Figure 4A). This response was

driven by *Cacna1c*$^{+/+}$ littermate controls but not *Cacna1c*$^{+/-}$ females, yet, characterized by a large variability between rats (t_{19}=1.226; p=0.235 and t_{19}=1.189; p=0.249; respectively). 22-kHz USV emission was low with less than one call per minute in the minutes following 50-kHz USV playback, irrespective of genotype (t_{19}=1.094; p=0.288 and t_{19}=1.426; p=0.170; respectively). USV emission in response to 50-kHz USV playback did not differ between genotypes (all p-values >0.050).

As in females, male receiver rats emitted USV in response to 50-kHz USV but not White Noise playback. Both *Cacna1c*$^{+/-}$ males and *Cacna1c*$^{+/+}$ littermate controls emitted more 50-kHz USV during 50-kHz USV playback than before (t_{19}=3.104; p=0.006 and t_{19}=2.270; p=0.035; respectively), again with less than one call per minute. While 50-kHz USV occurred only very rarely in response to 50-kHz USV playback, a substantial amount of 22-kHz USV was detected in both *Cacna1c*$^{+/-}$ males and *Cacna1c*$^{+/+}$ littermate controls (t_{19}=1.986; p=0.062 and t_{19}=3.329; p=0.004; respectively; Figure 4B). Moreover, and contrary to females, 22-kHz USV emission remained high following 50-kHz USV playback in both genotypes (t_{19}=2.116; p=0.048 and t_{19}=2.202; p=0.040; respectively). No genotype differences were detected (all p-values >0.050). When comparing female and male *Cacna1c*$^{+/+}$ littermate controls, USV in response to 50-kHz USV playback did not differ (all p-values >0.050). However, when comparing *Cacna1c*$^{+/-}$ females to male *Cacna1c*$^{+/+}$ littermate controls, 22-kHz USV but not 50-kHz USV in response to 50-kHz USV playback were particularly low in *Cacna1c*$^{+/-}$ females (t_{38}=3.112; p=0.004 and t_{38}=0.648; p=0.521).

Repetitive and stereotyped patterns of behavior

Repetitive behavior in females was not affected by genotype, with tail chasing (t_{38}=0.591; p=0.558; Suppl. Figure S2A) and self-grooming behavior being similar between genotypes (t_{38}=1.572; p=0.124; Suppl. Figure S2B), in line with findings obtained in male rats (Kisko et al., in press). Of note, locomotor activity during the assessment of repetitive and stereotyped patterns of behavior was not affected by genotype. Specifically, line crossings (t_{38}=0.657, p=0.515) and rearing events (t_{38}=0.631, p=0.532) occurred at similar levels in *Cacna1c*$^{+/-}$ females and *Cacna1c*$^{+/+}$ littermate controls.

Olfactory habituation and dishabituation

Finally, evidence for intact social and non-social olfactory abilities was obtained by means of the olfactory habituation and dishabituation paradigm, irrespective of genotype and sex (E: $F_{14,1064}$=4.375; p<0.001; ExG: $F_{14,1064}$=0.748; p=0.726; ExS: $F_{14,1064}$=0.671; p=0.805; ExGxS: $F_{14,1064}$=0.737; p=0.738). The expected zig-zag-shaped pattern was evident in *Cacna1c*$^{+/-}$ rats and *Cacna1c*$^{+/+}$ littermate controls, with rapid habituation occurring in response to the repeated exposure to social and non-social odor stimuli, but clear dishabituation between familiar and novel social and non-social odor stimuli (Suppl. Figure S3).

DISCUSSION

In this study, our aim was to explore the role of *Cacna1c* in regulating sex-specific effects in juvenile social behavior, specifically after weaning during the critical period of development in juvenile rats. *CACNA1C* mutations are strongly associated with multiple major neuropsychiatric disorders, including ASD, SCZ, MDD and (Splawski *et al.*, 2004, 2005, Sklar *et al.*, 2008, 2011; Ferreira *et al.*, 2008; Moskvina *et al.*, 2009; Dao *et al.*, 2010; Green *et al.*, 2010; Nyegaard *et al.*, 2010; Schizophrenia Psychiatric Genome-Wide Association Study (GWAS) Consortium, 2011; Ripke *et al.*, 2014; D'Gama *et al.*, 2015; Li *et al.*, 2015; Rao *et al.*, 2016). LTCC activity is also perturbed in a rare yet devastating disorder known as Timothy syndrome (TS), which has features partly similar to ASD (Barrett and Tsien, 2008). Most cases arise from a sporadic single nucleotide change that generates a missense mutation (G406R) in *CACNA1C* (Splawski *et al.*, 2004, 2005). Furthermore, SHANK scaffolding proteins, strongly associated with ASD (Monteiro and Feng, 2017), have been implicated in the regulation of LTCCs, and thus mutations in the *SHANK* gene family could lead to $Ca_v1.2$ malfunctioning (Pym *et al.*, 2017), possibly contributing to disorder-related behavioral phenotypes. In fact, a TS mouse model carrying the G406R replacement in $Ca_v1.2$ was reported to display a complete ASD-related behavioral phenotype characterized by lack of sociability, together with increased marble-burying behavior, impaired reversal learning abilities, and altered emission of isolation-induced ultrasonic calling in pups (Bader *et al.*, 2011) (but see (Kabitzke *et al.*, 2017)).

To our knowledge, however, disorder relevant phenotypes with bearing on socio-affective communication deficits in ASD, SCZ, and BPD have not been assessed in female rats with genetic modifications targeting *Cacna1c* until now. Moreover, available mouse studies almost exclusively focused on adult male mice, with no data being available for females (Jeon *et al.*, 2010; Bader *et al.*, 2011; Dedic *et al.*, 2017; Kabir *et al.*, 2017; Kabitzke *et al.*, 2017; Terrillion, Francis, *et al.*, 2017). Importantly, the role of *Cacna1c* in regulating socio-affective communication and disorder relevant behavioral phenotypes during the critical developmental period of adolescence, which is characterized by a particularly rich social behavior repertoire including social play behavior (Pellis and Pellis, 2009; Siviy and Panksepp, 2011; Vanderschuren, Achterberg and Trezza, 2016) has not yet been studied. Using our newly developed genetic *Cacna1c* rat model, we tested the hypothesis that female heterozygous *Cacna1c* rats show social behavior and communication impairments, together with repetitive and stereotyped patterns of behavior, when compared to female and male wildtype *Cacna1c*$^{+/+}$ littermate controls. Importantly, our assessment of communication impairments through pro-social 50-kHz USV (Portfors, 2007; Brudzynski, 2013; Wöhr and Schwarting, 2013) followed a truly communicative approach, including both sender and receiver.

Our results show for the first time that a deletion of *Cacna1c* leads to alterations in social behavior and pro-social 50-kHz ultrasonic communication in a sex-dependent manner in rats. In females, *Cacna1c*$^{+/-}$ rats spent more time playing than *Cacna1c*$^{+/+}$ littermate controls. Such genotype-dependent differences in social play behavior emerged across play sessions. While *Cacana1c*$^{+/-}$ females and *Cacna1c*$^{+/+}$ littermate controls did not differ in duration and numbers of playful events during the initial play session, *Cacna1c*$^{+/-}$ females increased the amount of time they spent playing to almost double that of *Cacna1c*$^{+/+}$ littermate controls during subsequent playful social interactions. Genotype differences were driven by elevated levels of pinning behavior, while wrestling and chasing were not strongly influenced by genotype. Importantly, genotype effects on social behavior were specifically seen in social play but not non-playful social behaviors, with individual aspects, such as sniffing and physical contact, not differing between *Cacna1c*$^{+/-}$ females and *Cacna1c*$^{+/+}$ littermate controls.

In contrast to social play behavior, concomitant emission of pro-social 50-kHz USV during social play behavior did not differ between genotypes. *Cacna1c*$^{+/-}$ and *Cacna1c*$^{+/+}$ females emitted comparable levels of 50-kHz USV while engaged in playful social interactions, however, in comparison to *Cacna1c*$^{+/+}$ males, female *Cacna1c*$^{+/-}$ and rats had lower 50-kHz USV emission rates. Since 50-kHz USV are believed to reflect positive affective states ("rat laughter"; (Panksepp, 2005)) associated with the rewarding nature of social play (Knutson, Burgdorf and Panksepp, 1998), this would suggest that playful encounters were similarly rewarding for *Cacna1c*$^{+/-}$ females as they were for *Cacna1c*$^{+/+}$ littermate controls. However, the substantial increase in pinning in *Cacna1c*$^{+/-}$ females does not support equivalent reward levels between the two genotypes but rather suggests that *Cacna1c*$^{+/-}$ females are compensating for the lack of reward by increasing rewarding behaviors. Additionally, the time spent pinning, after the initial play session in *Cacna1c*$^{+/-}$ females was higher even than *Cacna1c*$^{+/+}$ males, suggesting that they are not just expressing male-typical levels of playful interactions but are above the male-typical *Cacna1c*$^{+/+}$ littermate controls. Yet, interestingly, this was not reflected in 50-kHz USV emission. The similarity in 50-kHz USV emission between female *Cacna1c*$^{+/-}$ and *Cacna1c*$^{+/+}$ was evident across all playful social interactions, with genotypes not differing during subsequent play sessions, but with female *Cacna1c*$^{+/-}$ consistently remaining lower in comparison to male *Cacna1c*$^{+/+}$ 50-kHz USV emission levels. This means that 50-kHz USV were, likely, not driving the differences in social play behavior, but rather may imply that the playful interactions were not as rewarding for female *Cacna1c*$^{+/-}$ rats, reflected in reduced 50-kHz USV emission rates in comparison to male *Cacna1c*$^{+/+}$ rats.

It thus appears possible that *Cacna1c*$^{+/-}$ females may have compensated for low reward levels through increasing social play behavior, specifically pinning behavior. Using rough-and-tumble play rats can establish stable social relationships (Panksepp, 1981) and within social play pinning is hypothesized to provide an opportunity to maximize body-on-body contact during play fighting (Himmler *et al.*, 2016). In a study done by Kabitzke et al (2017), during a social recognition task male *Cacna1c*$^{+/-}$ mice spent considerably more time in close physical contact with each other compared to the wildtype controls, suggesting that the close nose-to-nape contact may be important for determining familiarity.

Although, Kabitzke et al (2017) tested only males, it is still possible *Cacna1c* haploinsufficiency also influences social recognition in females. Specifically, the fact that they strongly amplified the time they spend pinning, supports this view. Pinning and the close physical contact it offers, is arguably the most rewarding component of playful social interactions, as indicated by conditioned place preference (Vanderschuren, Achterberg and Trezza, 2016) and surgical devocalization (Kisko, Himmler, *et al.*, 2015; Kisko, Wöhr, *et al.*, 2015) experiments. Moreover, numerous studies have shown that rats find tickling by a human hand to be a highly rewarding experience, which stimulates them to emit high numbers of 50-kHz USV (Panksepp and Burgdorf, 2000, 2003; Burgdorf and Panksepp, 2001; Panksepp, 2007; Ishiyama and Brecht, 2016). Tickling is claimed to mimic the pinning sequence of playful social interactions; one rat lays supine and the hand, or other rat, takes an upright position above them and then "tickles" the abdomen, a particularly sensitive area (Panksepp and Burgdorf, 2003; Ishiyama and Brecht, 2016). Therefore, the more time spent pinning should naturally reinforce and increase the overall amount of reward to be gained from social play. Thus, the increase in 50-kHz USV emission by *Cacna1c*$^{+/-}$ females on the second and third play sessions, compared to baseline levels, is likely not a direct consequence of the increase in pinning behavior, but rather, is reflective of the overall increase in playful motivation across testing sessions, as this was also observed in *Cacna1c*$^{+/+}$ females. Interestingly, it has been shown that rats selectively bred for low levels of 50-kHz USV emission display altered *Cacna1c* gene expression together with ASD-like phenotypes and specifically during playful social interactions engage in more pinning behavior (Moskal *et al.*, 2011; Webber *et al.*, 2012; Burgdorf *et al.*, 2013).

Although the increase in pinning behavior was higher in *Cacna1c*$^{+/-}$ females than *Cacna1c*$^{+/+}$ littermate controls and concomitant 50-kHz USV emission increased across all three play sessions, it still only brought *Cacna1c*$^{+/-}$ females up to emission levels that are equal in comparison to the *Cacna1c*$^{+/+}$ littermate controls, yet lower than *Cacna1c*$^{+/+}$ males. This suggests that the higher duration of playful social interactions seen in *Cacna1c*$^{+/-}$ females on the second and third play sessions only grants them the same level of rewarding value gained by female *Cacna1c*$^{+/+}$ littermate controls. In other words, *Cacna1c*$^{+/-}$ females might need to

play more to get the same reward as *Cacna1c*$^{+/+}$ littermate controls, as indicated through 50-kHz USV emission rates. Importantly, *Cacna1c* deletion in females did not specifically affect the emission of 50-kHz USV during social play or 50-kHz USV throughout anticipation, suggesting that *Cacna1c* deletion in females affects the hedonic impact or *liking* of social reward, i.e. the pleasure derived from engaging in direct playful social contact (Berridge, Robinson and Aldridge, 2009), but only slightly the *wanting* component associated with playful social interactions.

In rats, sex differences in rough-and-tumble are widely reported (Pellis, 2002). In the current study, interestingly, however, no sex-differences were found between rough-and-tumble play of male and female *Cacna1c*$^{+/+}$ rats. Typically, male juvenile rats express a higher frequency of play (Thor and Holloway, 1983; Pellis and Pellis, 1990) and engage in rougher defense tactics i.e., more pinning, than females (Pellis and Pellis, 1990; Pellis, Pellis and McKenna, 1994). Yet, several studies, have shown that females can be manipulated to reflect more masculinized play patterns (Olioff and Stewart, 1978; Meaney and Stewart, 1981; Meaney and McEwen, 1986; Pellis and McKenna, 1995) and similarly, males can be made to mirror more female-typical play patterns (Beatty *et al.*, 1981; Meaney *et al.*, 1983; Ward and Stehm, 1991; Arnold and Siviy, 2002). For example, the introduction of testosterone neonatally (Olioff and Stewart, 1978; Thor and Holloway, 1983; Meaney and McEwen, 1986; Pellis and McKenna, 1995) creates a more masculinized playful repertoire in females. Likewise, blocking testosterone receptors in males creates female-typical play fighting (Meaney *et al.*, 1983). Our results in *Cacna1c*$^{+/-}$ females are suggestive for an enhanced and rougher male-typical play pattern, seen in the increase for the time spent pinning when compare to female and male Cacna*1c*$^{+/+}$ littermate controls. Additionally, the finding that *Cacna1c*$^{+/+}$ females do not differ form *Cacna1c*$^{+/+}$ males, suggests that there may be an environmental influence on the overall playful motivation of *Cacna1c*$^{+/+}$ females as a result of being housed with the *Cacna1c*$^{+/-}$ females, who show enhanced playful interactions. Indeed, some reports suggest that cagemates can indeed influence and affect playful motivations of each other (S. M. Himmler *et al.*, 2014; Kisko, Wöhr, *et al.*, 2015). For example, when housed with devocalized cagemates, intact, vocal, controls display severely decreased frequencies of playful interactions compared to controls housed with vocal cagemates (Kisko,

Wöhr, *et al.*, 2015). In terms of 50-kHz USV emission Himmler, Kisko et al (2014) reported a sex-specific effect in overall 50-kHz USV emission levels indicating that males typically emit more 50-kHz USV, supporting our findings that *Cacna1c*$^{+/+}$ males emit more 50-kHz USV than female *Cacna1c*$^{+/+}$ and *Cacna1c*$^{+/-}$ rats. A recent study by Lukas and Wöhr (2015) found that rats selectively bred for high, low and, non-selected anxiety levels, showed sex differences in social play, but no differences in 50-kHz USV emission. In both sexes higher anxiety resulted in frequencies of social play and 50-kHz USV. The playful frequencies of rats with low anxiety was comparable to the control, non-selected anxiety males, whereas in females, low anxiety resulted in a much higher frequency of playful interactions compared to control and high anxiety rats (Lukas and Wöhr, 2015). The findings of Lukas and Wöhr (2015) are particularly important because similar to our results, differences in social play in females did not result in changes to 50-kHz USV emission.

In *Cacna1c* rats, 50-kHz USV emission differed between the sexes, however unlike what was reported in *Cacna1c* males (Kisko et al, in press) there were no genotype differences between females in terms of 50-kHz USV emission. We previously observed that *Cacna1c*$^{+/-}$ males consistently emitted less 50-kHz USV while engaged in playful social interactions, suggesting an impairment in the *liking* aspect (Kisko et al, in press). In comparison however, in the *Cacna1c*$^{+/-}$ females even when rates of playful interactions differed from female *Cacna1c*$^{+/+}$ littermate controls, 50-kHz USV emission rates remained comparable between the genotypes. This, therefore, further indicates that Cacna*1c*$^{+/-}$ rats, irrespective of sex, may derive less reward from the playful interactions. Although, unlike the males *Cacna1c*$^{+/-}$ females appear to try and compensate for this by increasing the amount of time spent pinning. During the anticipation phase female *Cacna1c*$^{+/-}$ rats increased 50-kHz USV emission across repeated play sessions, which was not observed in *Cacna1c*$^{+/-}$ males, indicating that *Cacna1c* haploinsufficiency in females during playful interactions does not appear to fully affect *wanting,* but similar to males it may impact the *liking* component (Berridge, Robinson and Aldridge, 2009) associated with playful social interactions.

Unlike emission of 50-kHz USV in the sender, *Cacna1c* deletion in females slightly reduced the behavioral responses elicited by playback of 50-kHz USV. This

indicates that in females *Cacna1c* haploinsufficiency has detrimental effects on pro-social 50-kHz ultrasonic communication in the receiver. Which is reflected in a robust preference for the sound source, consistent with previous studies (Wöhr and Schwarting, 2007, 2009, 2012; Seffer *et al.*, 2015; Brenes *et al.*, 2016; Engelhardt *et al.*, 2017). Specifically, consistent with previous findings (Willadsen *et al.*, 2014), playback of pro-social 50-kHz USV led to social exploratory and approach behavior in females, with *Cacna1c*$^{+/-}$ females and *Cacna1c*$^{+/+}$ littermate controls displaying a clear preference for the sound source emitting 50-kHz USV. Importantly, in contrast to *Cacna1c* males (kisko et al, in press) the preference and strength of the behavioral response induced by 50-kHz USV playback was not affected by genotype. The increase in time spent in proximity to the sound source was similar in *Cacna1c*$^{+/+}$ littermate controls than in *Cacna1c*$^{+/-}$ females. Despite similar acute behavioral responses evoked by 50-kHz USV playback, however, evidence for genotype effects in the minutes following 50-kHz USV playback was obtained. While *Cacna1c*$^{+/+}$ littermate controls continued displaying a preference, no clear preference was seen in *Cacna1c*$^{+/-}$ females. This suggests that *Cacna1c*$^{+/+}$ littermate controls but not *Cacna1c*$^{+/-}$ females kept searching for a conspecific in proximity to the sound source after playback. Compared to *Cacna1c*$^{+/-}$ males, however, this is a relatively weak effect and consistent with the unaltered non-play social behavior in *Cacna1c*$^{+/-}$ females. Social approach behavior towards playback of pro-social 50-kHz USV reflects the motivation, i.e. *wanting*, for social contact, which is expressed in the amount of effort spent to obtain a social reward (Berridge, Robinson and Aldridge, 2009). Together with the increased social play, specifically pinning behavior and reduced 50-kHz USV emission rates in comparison to *Cacna1c*$^{+/+}$ males, this might indicate slight deficits in *wanting* in addition to impairments in the *liking* component associated with playful social interactions. Contrary to 50-kHz USV playback but in line with previous findings (Wöhr and Schwarting, 2012; Brenes *et al.*, 2016; Engelhardt *et al.*, 2017), White Noise led to strong behavioral inhibition in females, irrespective of genotype.

Reward processing (Schultz, 2002; Wise, 2004) and 50-kHz ultrasonic communication (Brudzynski, 2015; Rippberger *et al.*, 2015) have both been strongly associated with dopamine (DA). DA signaling is profoundly altered in mice with genetic modifications targeting *Cacna1c* (Kabir, Lee and Rajadhyaksha,

2016). It therefore appears possible that the observed impairments in social behavior and communication displayed by *Cacna1c*$^{+/-}$ rats are linked to deficits in DA signaling. Specifically, the emission of pro-social 50-kHz USV in the sender is critically dependent on DA signaling in the mesolimbic reward pathway. For instance, 50-kHz USV can be triggered by electrical stimulation of the medial forebrain bundle, including the ventral tegmental area (Burgdorf, Knutson and Panksepp, 2000; Burgdorf *et al.*, 2007; Scardochio *et al.*, 2015), with 50-kHz USV emission and phasic DA release in the nucleus accumbens being time-locked (Scardochio *et al.*, 2015). Moreover, 50-kHz USV emission can be induced pharmacologically by systemic administration of various psychostimulant drugs resulting in enhanced DA levels in the synaptic cleft, most notably amphetamine (Wright, Gourdon and Clarke, 2010; Simola *et al.*, 2012; Wright, Deng and Clarke, 2012; Pereira *et al.*, 2014; Wöhr *et al.*, 2015; Engelhardt *et al.*, 2017). Local administration of amphetamine in the nucleus accumbens was shown to result in a massive increase in 50-kHz USV emission (Burgdorf *et al.*, 2001; Thompson, Leonard and Brudzynski, 2006; Brudzynski *et al.*, 2011). Besides 50-kHz USV emission in the sender, social approach behavior elicited by playback of 50-kHz USV evokes phasic DA release in the nucleus accumbens of the receiver (Willuhn *et al.*, 2014) and enhancing DA signaling through the administration of amphetamine results in more pronounced 50-kHz USV responsivity (Engelhardt *et al.*, 2017). In mice with genetic modifications targeting *Cacna1c*, ample evidence indicates that DA signaling is impaired. For instance, Dao et al. (Dao *et al.*, 2010) found that hyperlocomotion induced by amphetamine treatment is reduced in adult male and female *Cacna1c*$^{+/-}$ mice as compared to *Cacna1c*$^{+/-}$ littermate controls. Moreover, Sittig et al. (Sittig *et al.*, 2016) observed reduced hyperlocomotion in response to methamphetamine in *Cacna1c*$^{+/-}$ mice on a wide range of genetic backgrounds. Terrillion et al. (Terrillion, Dao, *et al.*, 2017) extended these findings and showed that hyperlocomotion in adult male *Cacna1c*$^{+/-}$ mice is also reduced in response to cocaine and the specific DA transporter inhibitor GBR12909, yet not MK-801, indicating that specifically DA signaling is affected. By means of fast-scan cyclic voltammetry, they further showed that GBR12909 administration led to reduced extracellular DA concentrations in adult male *Cacna1c*$^{+/-}$ mice when compared to *Cacna1c*$^{+/-}$ littermate controls. This fits nicely with an electrophysiological study by Liu et al. (Liu *et al.*, 2014) demonstrating that $Ca_V1.2$

regulate DAergic burst firing in the ventral tegmental area. Together, this supports the idea that *Cacna1c* haploinsufficiency attenuates DA signaling in the mesolimbic reward pathway and is thus in line with reduced 50-kHz USV in male and female *Cacna1c*$^{+/-}$ rats. This is of considerable translational interest because the social motivation hypothesis of ASD states that atypical social behavior can be a result of the failure to assign reward values to social stimuli and interactions (Chevallier *et al.*, 2012). Moreover, a recent study by Panasiti et al. (Panasiti, Puzzo and Chakrabarti, 2016) found that ASD traits moderate the extent to which reward learning for social stimuli is transferred to pro-social behavior. Lower 50-kHz USV emission rates in female *Cacna1c*$^{+/+}$ rats may be explained by overall sex-differences in 50-kHz USV emission in rats during playful interactions (Himmler et al, 2014).

Importantly, in the present study, genotype effects on social behavior and response to ultrasonic communication were not due to impairments in behavioral activity and motor functions. Locomotor activity, rearing behavior, and climbing did not differ between genotypes. Moreover, confounding olfactory deficits can be ruled out, as evidenced in the olfactory habituation and dishabituation paradigm, with both genotypes showing the expected zig-zag-shaped pattern, reflecting intact olfactory abilities. This is consistent with previous findings in *Cacna1c*$^{+/-}$ mice by Dao et al. (Dao *et al.*, 2010). They showed that the time to find food in the hidden cookie test was not affected by *Cacna1c* haploinsufficiency. *Cacna1c*$^{+/-}$ rats further showed no repetitive behaviors, such as tail chasing or self-grooming, indicating that the deletion of *Cacna1c* does not result in the characteristic repetitive behaviors seen in human ASD (Battle, 2013) and relevant rodent models (Silverman *et al.*, 2010; Wöhr and Scattoni, 2013). This is in line with previous findings reporting a lack of repetitive and stereotyped patterns of behavior in *Cacna1c*$^{+/-}$ mice, as assessed by means of self-grooming (Lee *et al.*, 2012) and marble-burying (Bader *et al.*, 2011). This shows that *Cacna1c* haploinsufficiency specifically affected social behavior and communication among the ASD core symptoms and thus does not lead to a full ASD-related behavioral phenotype.

In humans, GWAS and other genetic approaches have identified a cluster of non-coding SNPs in *CACNA1C* to be strongly associated with multiple major neuropsychiatric disorders, including BPD, MDD, SCZ, and ASD (Splawski *et al.*,

2004, 2005, Sklar *et al.*, 2008, 2011; Ferreira *et al.*, 2008; Moskvina *et al.*, 2009; Dao *et al.*, 2010; Green *et al.*, 2010; Nyegaard *et al.*, 2010; Schizophrenia Psychiatric Genome-Wide Association Study (GWAS) Consortium, 2011; Ripke *et al.*, 2014; D'Gama *et al.*, 2015; Li *et al.*, 2015; Rao *et al.*, 2016). However, the mechanisms through which these SNPs confer susceptibility are not entirely clear. Roussos et al. (Roussos *et al.*, 2014) suggested that SNPs in the intronic region of the *CACNA1C* gene, such as rs1006737, could alter genome architecture and thus transcription by interacting with its transcription start site via chromosomal loopings. Indeed, there is some evidence that the *CACNA1C* risk variant rs1006737 and other SNPs affect pathophysiological pathways in associated neuropsychiatric disorders primarily by regulating protein expression without altering protein structure. For instance, the rs1006737 risk allele was found to be associated with decreased *CACNA1C* expression in the brains of SCZ (Roussos *et al.*, 2014) and BPD (Gershon *et al.*, 2014) patients. Moreover, *CACNA1C* hypermethylation was recently reported in BPD patients carrying the rs1006737 risk variant, suggesting that the regulatory effect of the non-coding risk variants involve a shift in DNA methylation, ultimately resulting in reduced protein expression (Starnawska *et al.*, 2016). Changes in the epigenetic regulation of *CACNA1C* were also linked to ASD (Sun *et al.*, 2016).

Importantly, as in our newly developed *Cacna1c* rat model characterized by a reduction of $Ca_v1.2$ protein expression, there is ample evidence suggesting altered social behavior and communication in human *CACNA1C* rs1006737 risk variant carriers. Firstly, the risk variant rs1006737 is associated with low extraversion in healthy individuals, a personality trait characterized by reduced preference for social activities and interactions (Roussos *et al.*, 2011), with genotype effects on personality traits being strongly sex-dependent (Strohmaier *et al.*, 2013). Secondly, the *CACNA1C* risk variant rs1006737 impairs socio-affective information processing in humans, slowing down facial emotion recognition in healthy individuals (Nieratschker, Brückmann and Plewnia, 2015) and reducing accuracy in BPD patients (Soeiro-de-Souza *et al.*, 2013). At the neurobiological level, rs1006737 risk allele carriers diagnosed with BPD are characterized by increased amygdala reactivity but decreased prefrontal activation during facial emotion processing (Bigos *et al.*, 2010; Jogia *et al.*, 2011; Tesli *et al.*, 2013), possibly due

to alterations in brain connectivity (Dima *et al.*, 2013; Radua *et al.*, 2013). Finally, verbal fluency is reduced in rs1006737 risk allele carriers, hindering language production on a semantic level (Krug *et al.*, 2010). Together, low extraversion with slowed and incorrect facial emotion recognition and impaired language production might thus impair social interaction and competence in human rs1006737 risk allele carriers. Interestingly, as suggested by the present and previous rodent findings (Dao *et al.*, 2010; Liu *et al.*, 2014; Sittig *et al.*, 2016; Terrillion, Dao, *et al.*, 2017), rs1006737 is also linked to alterations in reward processing in humans. For instance, Wessa et al. (Wessa *et al.*, 2010) observed increased amygdala reactivity in response to monetary reward in rs1006737 risk allele carriers. However, social reward has not yet been studied in humans.

CONCLUSION

In summary, our results show for the first time that a deletion of *Cacna1c* leads to alterations in social behavior and pro-social 50-kHz ultrasonic communication in a sex-dependent manner in rats. Increased playful interactions and specifically pinning behavior not paralleled by increased 50-kHz USV emission rates during rough-and-tumble play yet reduced in comparison to *Cacna1c*$^{+/+}$ males, suggest that female *Cacna1c* haploinsufficient rats derive less reward from playful social interactions and possibly adapt specific components, such as pinning, to compensate for the lack of reward. Besides emission of 50-kHz USV in the sender, in comparison to male *Cacna1c*$^{+/+}$ littermate controls, *Cacna1c* deletion slightly reduced the behavioral responses during the minutes following playback of 50-kHz USV. This indicates that *Cacna1c* haploinsufficiency has detrimental effects on pro-social 50-kHz ultrasonic communication in both, sender and receiver. Together, *Cacna1c* plays a prominent role in regulating sex-specific effects on pro-social behavior and socio-affective communication in rats with relevance for BPD, MDD, SCZ, and ASD.

DECLARATIONS:

List of Abbreviations: bipolar disorder (BPD); major depressive disorder (MDD); schizophrenia (SCZ); autism spectrum disorder (ASD); voltage-gated L-type calcium channel (LTCC); single-nucleotide polymorphism (SNP); genome-wide association study (GWAS); heterozygous *Cacna1c* (*Cacna1c*$^{+/-}$); wildtype *Cacna1c* (*Cacna1c*$^{+/+}$); postnatal day (PND), Sprague-Dawley (SD), ultrasonic vocalizations (USV).

Ethics Approval and Consent to Participate: All procedures were conducted in strict accordance with the the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and the relevant local or national rules and regulations of Germany and were subject to prior authorization by the local government (MR 20/35 Nr. 19/2014; Tierschutzbehörde, Regierungspräsdium Gießen, Germany).

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REFERENCES

Arnold, J. L. and Siviy, S. M. (2002) 'Effects of neonatal handling and maternal separation on rough-and-tumble play in the rat', *Developmental Psychobiology*, 41(3), pp. 205–215.

Bader, P. L., Faizi, M., Kim, L. H., Owen, S. F., Tadross, M. R., Alfa, R. W., Bett, G. C. L., Tsien, R. W., Rasmusson, R. L. and Shamloo, M. (2011) 'Mouse model of Timothy syndrome recapitulates triad of autistic traits', *Proceedings of the National Academy of Sciences*, 108(37), pp. 15432–15437. doi: 10.1073/pnas.1112667108.

Baenninger, L. P. (1966) 'The reliability of dominance orders in rats.', *Animal behaviour*, 14(2–3), pp. 367–371. doi: 10.1016/S0003-3472(66)80099-4.

Barrett, C. F. and Tsien, R. W. (2008) 'The Timothy syndrome mutation differentially affects voltage- and calcium-dependent inactivation of Ca(V)1.2 L-type calcium channels', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 105(6), pp. 2157–2162. doi: 10.1073/pnas.0710501105.

Battle, D. E. (2013) 'Diagnostic and Statistical Manual of Mental Disorders (DSM).', *CoDAS*, 25(2), pp. 191–192. doi: 10.1176/appi.books.9780890425596.744053.

Beatty, W. W., Dodge, A. M., Traylor, K. L. and Meaney, M. J. (1981) 'Temporal boundary of the sensitive period for hormonal organization of social play in juvenile rats', *Physiology & behavior*. 26(2), pp. 241–243.

Berridge, K. C., Robinson, T. E. and Aldridge, J. W. (2009) 'Dissecting components of reward: "liking", "wanting", and learning', *Current opinion in pharmacology*, 9(1), pp. 65–73. doi: 10.1016/j.coph.2008.12.014.

Bhat, S., Dao, D. T., Terrillion, C. E., Arad, M., Smith, R. J., Soldatov, N. M. and Gould, T. D. (2012) 'CACNA1C (Ca v 1.2) in the pathophysiology of psychiatric disease', *Progress in Neurobiology*, 99, pp. 1–14. doi: 10.1016/j.pneurobio.2012.06.001.

Bigos, K. L., Mattay, V. S., Callicott, J. H., Straub, R. E., Vakkalanka, R., Kolachana, B., Hyde, T. M., Lipska, B. K., Kleinman, J. E. and Weinberger, D. R. (2010) 'Genetic Variation in CACNA1C Affects Brain Circuitries Related to Mental Illness', *Arch Gen Psychiatry*, 67(9), pp. 939–945. doi: 10.1017/CBO9781107415324.004.

Bolles, R. C. and Woods, P. J. (1964) 'The ontogeny of behaviour in the albino rat', *Animal Behaviour*, 12(4), pp. 427–441. doi: 10.1016/0003-3472(64)90062-4.

Brenes, J. C., Lackinger, M., Höglinger, G. U., Schratt, G., Schwarting, R. K. W. and Wöhr, M. (2016) 'Differential effects of social and physical environmental enrichment on brain plasticity, cognition, and ultrasonic communication in rats', *Journal of Comparative Neurology*, 524(8), pp. 1586–1607. doi: 10.1002/cne.23842.

Brudzynski, S. M. (2013) 'Ethotransmission: Communication of emotional states through ultrasonic vocalization in rats', *Current Opinion in Neurobiology*, pp. 310–317.

Brudzynski, S. M. (2015) 'Pharmacology of Ultrasonic Vocalizations in adult Rats: Significance, Call Classification and Neural Substrate', *Current Neuropharmacology*. Bentham Science Publishers, 13(2), pp. 180–192. doi: 10.2174/1570159X13999150210141444.

Brudzynski, S. M. and Pniak, A. (2002) 'Social contacts and production of 50-kHz short ultrasonic calls in adult rats', *Journal of comparative psychology, 116(1),* pp. 73-82. doi: 10.1037/0735-7036.116.1.73.

Brudzynski, S. M., Silkstone, M., Komadoski, M., Scullion, K., Duffus, S., Burgdorf, J., Kroes, R. A., Moskal, J. R. and Panksepp, J. (2011) 'Effects of intraaccumbens amphetamine on production of 50kHz vocalizations in three lines of selectively bred Long-Evans rats', *Behavioural Brain Research*, 217(1), pp. 32–40. doi: 10.1016/j.bbr.2010.10.006.

Burgdorf, J., Knutson, B. and Panksepp, J. (2000) 'Anticipation of rewarding electrical brain stimulation evokes ultrasonic vocalization in rats', *Behavioral Neuroscience*, 114(2), pp. 320–327. doi: 10.1037//0735-7044.114.2.320.

Burgdorf, J., Knutson, B., Panksepp, J. and Ikemoto, S. (2001) 'Nucleus accumbens amphetamine microinjections unconditionally elicit 50-kHz ultrasonic vocalizations in rats', *Behavioral Neuroscience*, 115(4), pp. 940–944. doi: 10.1037/0735-7044.115.4.940.

Burgdorf, J., Moskal, J. R., Brudzynski, S. M. and Panksepp, J. (2013) 'Rats selectively bred for low levels of play-induced 50kHz vocalizations as a model for Autism Spectrum Disorders: A role for NMDA receptors', *Behavioural Brain Research*, 251, pp. 18–24. doi: 10.1016/j.bbr.2013.04.022.

Burgdorf, J. and Panksepp, J. (2001) 'Tickling induces reward in adolescent rats', *Physiology & Behavior*, 72(1–2), pp. 167–173. doi: 10.1016/S0031-9384(00)00411-X.

Burgdorf, J., Wood, P. L., Kroes, R. A., Moskal, J. R. and Panksepp, J. (2007) 'Neurobiology of 50-kHz ultrasonic vocalizations in rats: Electrode mapping, lesion, and pharmacology studies', *Behavioural Brain Research*, 182(2), pp. 274–283.

Chevallier, C., Kohls, G., Troiani, V., Brodkin, E. S. and Schultz, R. T. (2012) 'The social motivation theory of autism', *Trends in Cognitive Sciences*, 16(4), pp. 231–239.

Consortium, C.-D. G. of the P. G. (2013) 'Identification of risk loci with shared effects on five major psychiatric disorders: a genome-wide analysis', *Lancet*, 981(9875), pp. 1371-1379. doi: 10.1016/S0140-6736(12)62129-1.

D'Gama, A. M., Pochareddy, S., Li, M., Jamuar, S. S., Reiff, R. E., Lam, A.-T. N., Sestan, N. and Walsh, C. A. (2015) 'Targeted DNA Sequencing from Autism Spectrum Disorder Brains Implicates Multiple Genetic Mechanisms', *Neuron*, 88(5), pp. 910–917. doi: 10.1016/j.neuron.2015.11.009.

Dao, D. T., Mahon, P. B., Cai, X., Kovacsics, C. E., Blackwell, R. A., Arad, M., Shi, J., Zandi, P. P., O'Donnell, P., Knowles, J. A., Weissman, M. M., Coryell, W., Scheftner, W. A., Lawson, W. B., Levinson, D. F., Thompson, S. M., Potash, J. B. and Gould, T. D. (2010) 'Mood disorder susceptibility gene CACNA1C modifies mood-related behaviors in mice and interacts with sex to influence behavior in mice and diagnosis in humans', *Biological Psychiatry, 68(9)*, pp. 801-810. doi: 10.1016/j.biopsych.2010.06.019.

Dedic, N., Pohlmann, M. L., Richter, J. S., Mehta, D., Czamara, D., Metzger, M. W., Dine, J., Bedenk, B. T., Hartmann, J., Wagner, K. V, Jurik, A., Almli, L. M., Lori, A., Moosmang, S., Hofmann, F., Wotjak, C. T., Rammes, G., Eder, M., Chen, A.,

Ressler, K. J., Wurst, W., Schmidt, M. V, Binder, E. B. and Deussing, J. M. (2017) 'Cross-disorder risk gene CACNA1C differentially modulates susceptibility to psychiatric disorders during development and adulthood', *Molecular Psychiatry,* 23(3), pp. 533-543.

Dima, D., Jogia, J., Collier, D., Vassos, E., Burdick, K. E. and Frangou, S. (2013) 'Independent Modulation of Engagement and Connectivity of the Facial Network During Affect Processing by CACNA1C and ANK3 Risk Genes for Bipolar Disorder', *JAMA Psychiatry*, 70(12), pp. 1303. doi: 10.1001/jamapsychiatry.2013.2099.

Ellenbroek, B. and Youn, J. (2016) 'Rodent models in neuroscience research: is it a rat race?', *Disease Models & Mechanisms*, 9(10), pp. 1079–1087. doi: 10.1242/dmm.026120.

Endres, T., Widmann, K. and Fendt, M. (2007) 'Are rats predisposed to learn 22kHz calls as danger-predicting signals?', *Behavioural Brain Research*, 185(2), pp. 69–75.

Engelhardt, K. A., Fuchs, E., Schwarting, R. K. W. and Wöhr, M. (2017) 'Effects of amphetamine on pro-social ultrasonic communication in juvenile rats: Implications for mania models', *European Neuropsychopharmacology*, 27(3), pp. 261–273.

Ferreira, M. A., O'Donovan, M. C., Meng, Y. A., Jones, I. R., Ruderfer, D. M., Jones, L., Fan, J., Kirov, G., Perlis, R. H., Green, E. K., Smoller, J. W., Grozeva, D., Stone, J., Nikolov, I., Chambert, K., Hamshere, M. L., Nimgaonkar, V. L., Moskvina, V., Thase, M. E., Caesar, S., Sachs, G. S., Franklin, J., Gordon-Smith, K., Ardlie, K. G., Gabriel, S. B., Fraser, C., Blumenstiel, B., Defelice, M., Breen, G. and Gill, M. (2008) 'Collaborative genome-wide association analysis supports a role for ANK3 and CACNA1C in bipolar disorder', *Nature Genetetics*, 40(9), pp. 1056-1058. doi: 10.1038/ng.209.

Gershon, E. S., Grennan, K., Busnello, J., Badner, J. A., Ovsiew, F., Memon, S., Alliey-Rodriguez, N., Cooper, J., Romanos, B. and Liu, C. (2014) 'A rare mutation of CACNA1C in a patient with bipolar disorder, and decreased gene expression associated with a bipolar-associated common SNP of CACNA1C in brain.', *Molecular psychiatry*, 19, pp. 890-894. doi: 10.1038/mp.2013.107.

Geurts, A. M., Cost, G. J., Freyvert, Y., Zeitler, B., Miller, J. C., Choi, V. M., Jenkins, S. S., Wood, A., Cui, X., Meng, X., Vincent, A., Lam, S., Michalkiewicz, M., Schilling, R., Foeckler, J., Kalloway, S., Weiler, H., Ménoret, S., Anegon, I., Davis, G. D., Zhang, L., Rebar, E. J., Gregory, P. D., Urnov, F. D., Jacob, H. J. and Buelow, R. (2009) 'Knockout Rats via Embryo Microinjection of Zinc-Finger Nucleases', *Science*, 325(5939), pp. 433-433.

Green, E. K., Grozeva, D., Jones, I., Jones, L., Kirov, G., Caesar, S., Gordon-Smith, K., Fraser, C., Forty, L., Russell, E., Hamshere, M. L., Moskvina, V., Nikolov, I., Farmer, A., McGuffin, P., Holmans, P. A., Owen, M. J., O'Donovan, M. C. and Craddock, N. (2010) 'The bipolar disorder risk allele at CACNA1C also confers risk of recurrent major depression and of schizophrenia', *Molecular Psychiatry*, 15, pp. 1016-1022. doi: 10.1038/mp.2009.49.

Heyes, S., Pratt, W. S., Rees, E., Dahimene, S., Ferron, L., Owen, M. J. and Dolphin, A. C. (2015) 'Genetic disruption of voltage - gated calcium channels in psychiatric and neurological disorders.', *Progress in neurobiology*, *134*, 36-54.

Himmler, B. T., Kisko, T. M., Euston, D. R., Kolb, B. and Pellis, S. M. (2014) 'Are 50-kHz calls used as play signals in the playful interactions of rats? I. Evidence from the timing and context of their use', *Behavioural Processes*, 106, pp. 60–66.

Himmler, S. M., Himmler, B. T., Stryjek, R., Modlińska, K., Pisula, W. and Pellis, S. M. (2016) 'Pinning in the play fighting of rats: A comparative perspective with methodological recommendations', *Journal Issue: International Journal of Comparative Psychology, 29*.

Himmler, S. M., Modlinska, K., Stryjek, R., Himmler, B. T., Pisula, W. and Pellis, S. M. (2014) 'Domestication and diversification: A comparative analysis of the play fighting of the Brown Norway, Sprague-Dawley, and Wistar laboratory strains of (Rattus norvegicus).', *Journal of Comparative Psychology*, 128(3), pp. 318–327. doi: 10.1037/a0036104.

Homberg, J. R., Schiepers, O. J. G., Schoffelmeer, A. N. M., Cuppen, E. and Vanderschuren, L. J. (2007) 'Acute and constitutive increases in central serotonin levels reduce social play behaviour in peri-adolescent rats.', *Psychopharmacology*, 195(2), pp. 175–82. doi: 10.1007/s00213-007-0895-8.

Homberg, J. R., Wöhr, M. and Alenina, N. (2017) 'Comeback of the Rat in Biomedical Research', *ACS Chemical Neuroscience*, 8(5), pp. 900–903. doi: 10.1021/acschemneuro.6b00415.

Ishiyama, S. and Brecht, M. (2016) 'Neural correlates of ticklishness in the rat somatosensory cortex', *Science*, 354(6313), pp. 757 LP-760.

Jeon, D., Kim, S., Chetana, M., Jo, D., Ruley, H. E., Lin, S.-Y., Rabah, D., Kinet, J.-P. and Shin, H.-S. (2010) 'Observational fear learning involves affective pain system and Cav1.2 Ca2+ channels in ACC', *Nature Neuroscience*, 13(4), pp. 482–488. doi: 10.1038/nn.2504.

Jogia, J., Ruberto, G., Lelli-Chiesa, G., Vassos, E., Maierú, M., Tatarelli, R., Girardi, P., Collier, D. and Frangou, S. (2011) 'The impact of the CACNA1C gene polymorphism on frontolimbic function in bipolar disorder', *Molecular Psychiatry*, 16(11), pp. 1070–1071. doi: 10.1038/mp.2011.49.

Kabir, Z. D., Che, A., Fischer, D. K., Rice, R. C., Rizzo, B. K., Byrne, M., Glass, M. J., De Marco Garcia, N. V and Rajadhyaksha, A. M. (2017) 'Rescue of impaired sociability and anxiety-like behavior in adult cacna1c-deficient mice by pharmacologically targeting eIF2α', *Molecular Psychiatry*, 22(8), pp. 1096–1109. doi: 10.1038/mp.2017.124.

Kabir, Z. D., Lee, A. S. and Rajadhyaksha, A. M. (2016) 'L-type Ca2+ channels in mood, cognition and addiction: integrating human and rodent studies with a focus on behavioural endophenotypes', *The Journal of Physiology*, 594(20), pp. 5823–5837. doi: 10.1113/JP270673.

Kabitzke, P., Brunner, D., He, D., Fazio, P. A., Cox, K., Sutphen, J., Thiede, L., Sabath, E., Hanania, T., Alexandrov, V., Rasmusson, R., Spooren, W., Ghosh, A., Feliciano, P., Biemans, B., Benedetti, M. and Luo Clayton, A. (2017) 'Comprehensive Analysis of Two Shank3 and the Cacna1c Mouse Models of Autism Spectrum Disorder.', *Genes, brain, and behavior*, 17(1) pp. 1–36. doi: 10.1111/gbb.12405.

Kisko, T. M., Braun, M. D., Michels, S., Witt, S. H., Rietschel, M., Culmsee, C., Schwarting, R. K. W., Wöhr, M. (in press). '*Cacna1c* haploinsufficiency leads to pro-social 50-kHz ultrasonic communication deficits in rats'. *Disease Models and Mechanisms*.

Kisko, T. M., Himmler, B. T., Himmler, S. M., Euston, D. R. and Pellis, S. M. (2015) 'Are 50-kHz calls used as play signals in the playful interactions of rats? II. Evidence from the effects of devocalization', *Behavioural Processes*, 111. doi: 10.1016/j.beproc.2014.11.011.

Kisko, T. M., Wöhr, M., Pellis, V. C. and Pellis, S. M. (2015) 'From Play to Aggression: High-Frequency 50-kHz Ultrasonic Vocalizations as Play and Appeasement Signals in Rats', in Wöhr, M. and Krach, S. (eds) *Current Topics in Behavioral Neurosciences*. Switzerland: Springer International Publishing, pp. 91–108. doi: 10.1007/7854_2015_432.

Knutson, B., Burgdorf, J. and Panksepp, J. (1998) 'Anticipation of play elicits high-frequency ultrasonic vocalizations in young rats.', *Journal of Comparative Psychology*, 112(1), pp. 65–73. doi: 10.1037/0735-7036.112.1.65.

Krug, A., Nieratschker, V., Markov, V., Krach, S., Jansen, A., Zerres, K., Eggermann, T., Stöcker, T., Shah, N. J., Treutlein, J., Mühleisen, T. W. and Kircher, T. (2010) 'Effect of CACNA1C rs1006737 on neural correlates of verbal fluency in healthy individuals', *NeuroImage*. doi: 10.1016/j.neuroimage.2009.09.028.

Lee, A. S., Ra, S., Rajadhyaksha, A. M., Britt, J. K., De Jesus-Cortes, H., Gonzales, K. L., Lee, A., Moosmang, S., Hofmann, F., Pieper, a a and Rajadhyaksha, A. M. (2012) 'Forebrain elimination of cacna1c mediates anxiety-like behavior in mice', *Molecular Psychiatry*, 17(11), pp. 1054–1055. doi: 10.1038/mp.2012.71.

Li, J., Zhao, L., You, Y., Lu, T., Jia, M., Yu, H., Ruan, Y., Yue, W., Liu, J., Lu, L., Zhang, D. and Wang, L. (2015) 'Schizophrenia related variants in CACNA1C also confer risk of autism', *PLoS ONE*. doi: 10.1371/journal.pone.0133247.

Liu, Y., Blackwood, D. H., Caesar, S., Geus, E. J. C. de, Farmer, A., Ferreira, M. a. R., Ferrier, I. N., Fraser, C., Gordon-Smith, K., Green, E. K., Grozeva, D., Gurling, H. M., Hamshere, M. L., Heutink, P., Holmans, P. A., Hoogendijk, W. J., Hottenga, J. J., Jones, L., Jones, I. R., Kirov, G., Lin, D., McGuffin, P., Moskvina, V., Nolen, W. A., Perlis, R. H., Posthuma, D., Scolnick, E. M., Smit, A. B., Smit, J. H., Smoller, J. W., Clair, D. S., Dyck, R. van, Verhage, M., Willemsen, G., Young, A. H., Zandbelt, T., Boomsma, D. I., Craddock, N., O'Donovan, M. C., Owen, M. J., Penninx, B. W. J. H., Purcell, S., Sklar, P. and Sullivan, P. F. (2011) 'Meta-analysis of genome-wide association data of bipolar disorder and major depressive disorder', *Molecular Psychiatry*, 16, pp. 2-4. doi: 10.1038/mp.2009.107.

Liu, Y., Harding, M., Pittman, A., Dore, J., Striessnig, J., Rajadhyaksha, A. and Chen, X. (2014) 'Cav1.2 and Cav1.3 L-type calcium channels regulate dopaminergic firing activity in the mouse ventral tegmental area', *Journal of Neurophysiology*, 112(5), pp. 1119–1130. doi: 10.1152/jn.00757.2013.

Lukas, M. and Wöhr, M. (2015) 'Endogenous vasopressin, innate anxiety, and the emission of pro-social 50-kHz ultrasonic vocalizations during social play behavior in juvenile rats', *Psychoneuroendocrinology*, 56, pp. 35–44. doi: 10.1016/j.psyneuen.2015.03.005.

Meaney, M. J. and McEwen, B. S. (1986) 'Testosterone implants into the amygdala during the neonatal period masculinize the social play of juvenile female rats', *Brain Research*. Elsevier, 398(2), pp. 324–328. doi: 10.1016/0006-8993(86)91492-7.

Meaney, M. J. and Stewart, J. (1981) 'A descriptive study of social development in the rat (Rattus norvegicus)', *Animal Behaviour*, 29(1), pp. 34–45. doi: 10.1016/S0003-3472(81)80149-2.

Meaney, M. J., Stewart, J., Poulin, P. and McEwen, B. S. (1983) 'Sexual differentiation of social play in rat pups is mediated by the neonatal androgen-receptor system', *Neuroendocrinology*, 37(2), pp. 85–90.

Meyer-Lindenberg, A. and Tost, H. (2012) 'Neural mechanisms of social risk for psychiatric disorders.', *Nature neuroscience*, 15(5), pp. 663–8. doi: 10.1038/nn.3083.

Monteiro, P. and Feng, G. (2017) 'SHANK proteins: roles at the synapse and in autism spectrum disorder', *Nature Reviews Neuroscience*, 18(3), pp. 147–157. doi: 10.1038/nrn.2016.183.

Moskal, J. R., Burgdorf, J., Kroes, R. A., Brudzynski, S. M. and Panksepp, J. (2011) 'A novel NMDA receptor glycine-site partial agonist, GLYX-13, has therapeutic potential for the treatment of autism', *Neuroscience and Biobehavioral Reviews*, 35(9), pp. 1982–1988. doi: 10.1016/j.neubiorev.2011.06.006.

Moskvina, V., Craddock, N., Holmans, P., Nikolov, I., Pahwa, J. S., Green, E., Owen, M. J. and O'Donovan, M. C. (2009) 'Gene-wide analyses of genome-wide association data sets: evidence for multiple common risk alleles for schizophrenia and bipolar disorder and for overlap in genetic risk', *Molecular Psychiatry*, 14. doi: 10.1038/mp.2008.133.

Nieratschker, V., Brückmann, C. and Plewnia, C. (2015) 'CACNA1C risk variant affects facial emotion recognition in healthy individuals', *Scientific Reports*, 5. doi: 10.1038/srep17349.

Nyegaard, M., Demontis, D., Foldager, L., Hedemand, A., Flint, T. J., Sørensen, K. M., Andersen, P. S., Nordentoft, M., Werge, T., Pedersen, C. B., Hougaard, D. M., Mortensen, P. B., Mors, O. and Børglum, A. D. (2010) 'CACNA1C (rs1006737) is associated with schizophrenia', *Molecular Psychiatry, 15,* pp. 119-121. doi: 10.1038/mp.2009.69.

Olioff, M. and Stewart, J. (1978) 'Sex differences in the play behavior of prepubescent rats', *Physiology & Behavior*, 20(2), pp. 113–115.

Ou, X., Crane, D. E., MacIntosh, B. J., Young, L. T., Arnold, P., Ameis, S. and Goldstein, B. I. (2015) 'CACNA1C rs1006737 genotype and bipolar disorder: Focus on intermediate phenotypes and cardiovascular comorbidity', *Neuroscience and Biobehavioral Reviews*, 55, pp. 198-210. doi: 10.1016/j.neubiorev.2015.04.022.

Panasiti, M. S., Puzzo, I. and Chakrabarti, B. (2016) 'Autistic Traits Moderate the Impact of Reward Learning on Social Behaviour', *Autism Research*, 9(4), pp. 471–479. doi: 10.1002/aur.1523.

Panksepp, J. (1981) 'The ontogeny of play in rats', *Developmental Psychobiology*, 14(4), pp. 327–332. doi: 10.1002/dev.420140405.

Panksepp, J. (2005) ‘Beyond a Joke: From Animal Laughter to Human Joy?’, *Science*, 308(5718), pp. 62-63.

Panksepp, J. (2007) ‘Neuroevolutionary sources of laughter and social joy: Modeling primal human laughter in laboratory rats’, *Behavioural brain research*, 182(2), pp. 231–244.

Panksepp, J. and Burgdorf, J. (2000) ‘50-kHz chirping (laughter?) in response to conditioned and unconditioned tickle-induced reward in rats: effects of social housing and genetic variables’, *Behavioural brain research*, 115(1), pp. 25–38.

Panksepp, J. and Burgdorf, J. (2003) ‘“Laughing” rats and the evolutionary antecedents of human joy?’, *Physiology and Behavior*, 79(3), pp. 533–547. doi: 10.1016/S0031-9384(03)00159-8.

Panksepp, J., Gordon, N. and Burgdorf, J. (2002) ‘Empathy and the action-perception resonances of basic socio-emotional systems of the brain’, *Behavioral and Brain Sciences, 25(1)*, pp. 43–44. doi: 10.1017/S0140525X0247001X.

Pellis, S. M. (2002) ‘Sex Differences in Play Fighting Revisited: Traditional and Nontraditional Mechanisms of Sexual Differentiation in Rats’, *Archives of Sexual Behavior*, 31(1), pp. 17–26. doi: 10.1023/A:1014070916047.

Pellis, S. M. and McKenna, M. (1995) ‘What do rats find rewarding in play fighting?—an analysis using drug-induced non-playful partners’, *Behavioural brain research*, 68(1), pp. 65–73.

Pellis, S. M. and Pellis, V. C. (1990) ‘Differential rates of attack, defense, and counterattack during the developmental decrease in play fighting by male and female rats’, *Developmental Psychobiology*, 23(3), pp. 215–231. doi: 10.1002/dev.420230303.

Pellis, S. M. and Pellis, V. C. (2009) *The playful brain: venturing to the limits of neuroscience*. Oneworld Publications.

Pellis, S. M., Pellis, V. C. and McKenna, M. M. (1994) ‘Feminine dimension in the play fighting of rats (Rattus norvegicus) and its defeminization neonatally by androgens.’, *Journal of Comparative Psychology*, 108(1), pp. 68.

Pereira, M., Andreatini, R., Schwarting, R. K. W. and Brenes, J. C. (2014) ‘Amphetamine-induced appetitive 50-kHz calls in rats: a marker of affect in mania?’, *Psychopharmacology*, 231(13), pp. 2567–2577. doi: 10.1007/s00213-013-3413-1.

Portfors, C. V (2007) ‘Types and functions of ultrasonic vocalizations in laboratory rats and mice.’, *Journal of the American Association for Laboratory Animal Science : JAALAS*, 46(1), pp. 28–34.

Pym, E., Sasidharan, N., Thompson-Peer, K. L., Simon, D. J., Anselmo, A., Sadreyev, R. I., Hall, Q., Nurrish, S. and Kaplan, J. M. (2017) ‘Shank is a dose-dependent regulator of Cav1 calcium current and CREB target expression’, *eLife*, 6, pp. e18931.

Radua, J., Surguladze, S. A., Marshall, N., Walshe, M., Bramon, E., Collier, D. A., Prata, D. P., Murray, R. M. and McDonald, C. (2013) ‘The impact of CACNA1C allelic variation on effective connectivity during emotional processing in bipolar disorder’, *Molecular psychiatry*, 18(5), p. 526. doi: 10.1038/mp.2012.61.

Rao, S., Yao, Y., Zheng, C., Ryan, J., Mao, C., Zhang, F., Meyre, D. and Xu, Q. (2016) ‘Common variants in CACNA1C and MDD susceptibility: A comprehensive meta-

analysis', *American Journal of Medical Genetics, Part B: Neuropsychiatric Genetics*, 171(6), pp. 896–903. doi: 10.1002/ajmg.b.32466.

Raza, S., Himmler, B. T., Himmler, S. M., Harker, A., Kolb, B., Pellis, S. M. and Gibb, R. (2015) 'Effects of prenatal exposure to valproic acid on the development of juvenile-typical social play in rats.', *Behavioural Pharmacology*, 26(8), pp. 707–19.

Ripke, S., Neale, B. M., Corvin, A., Walters, J. T. R., Farh, K.-H., Holmans, P. a., Lee, P., Bulik-Sullivan, B., Collier, D. a., Huang, H., Pers, T. H., Agartz, I., Agerbo, E., Albus, M., Alexander, M., Amin, F., Bacanu, S. a., Begemann, M., Belliveau Jr, R. a., Bene, J., Bergen, S. E., Bevilacqua, E., Bigdeli, T. B., Black, D. W., Bruggeman, R., Buccola, N. G., Buckner, R. L., Byerley, W., Cahn, W., Cai, G., Campion, D., Cantor, R. M., Carr, V. J., Carrera, N., Catts, S. V., Chambert, K. D., Chan, R. C. K., Chen, R. Y. L., Chen, E. Y. H., Cheng, W., Cheung, E. F. C., Ann Chong, S., Robert Cloninger, C., Cohen, D., Cohen, N., Cormican, P., Craddock, N., Crowley, J. J., Curtis, D., Davidson, M., Davis, K. L., Degenhardt, F., Del Favero, J., Demontis, D., Dikeos, D., Dinan, T., Djurovic, S., Donohoe, G., Drapeau, E., Duan, J., Dudbridge, F., Durmishi, N., Eichhammer, P., Eriksson, J., Escott-Price, V., Essioux, L., Fanous, A. H., Farrell, M. S., Frank, J., Franke, L., Freedman, R., Freimer, N. B., Friedl, M., Friedman, J. I., Fromer, M., Genovese, G., Georgieva, L., Giegling, I., Giusti-Rodríguez, P., Godard, S., Goldstein, J. I., Golimbet, V., Gopal, S., Gratten, J., de Haan, L., Hammer, C., Hamshere, M. L., Hansen, M., Hansen, T., Haroutunian, V., Hartmann, A. M., Henskens, F. a., Herms, S., Hirschhorn, J. N., Hoffmann, P., Hofman, A., Hollegaard, M. V., Hougaard, D. M., Ikeda, M., Joa, I., Julià, A., Kahn, R. S., Kalaydjieva, L., Karachanak-Yankova, S., Karjalainen, J., Kavanagh, D., Keller, M. C., Kennedy, J. L., Khrunin, A., Kim, Y., Klovins, J., Knowles, J. a., Konte, B., Kucinskas, V., Ausrele Kucinskiene, Z., Kuzelova-Ptackova, H., Kähler, A. K., Laurent, C., Lee Chee Keong, J., Hong Lee, S., Legge, S. E., Lerer, B., Li, M., Li, T., Liang, K.-Y., Lieberman, J., Limborska, S., Loughland, C. M., Lubinski, J., Lönnqvist, J., Macek Jr, M., Magnusson, P. K. E., Maher, B. S., Maier, W., Mallet, J., Marsal, S., Mattheisen, M., Mattingsdal, M., McCarley, R. W., McDonald, C., McIntosh, A. M., Meier, S., Meijer, C. J., Melegh, B., Melle, I., Mesholam-Gately, R. I., Metspalu, A., Michie, P. T., Milani, L., Milanova, V., Mokrab, Y., Morris, D. W., Mors, O., Murphy, K. C., Murray, R. M., Myin-Germeys, I., Müller-Myhsok, B., Nelis, M., Nenadic, I., Nertney, D. a., Nestadt, G., Nicodemus, K. K., Nikitina-Zake, L., Nisenbaum, L., Nordin, A., O'Callaghan, E., O'Dushlaine, C., O'Neill, F. A., Oh, S.-Y., Olincy, A., Olsen, L., Van Os, J., Endophenotypes International Consortium, P., Pantelis, C., Papadimitriou, G. N., Papiol, S., Parkhomenko, E., Pato, M. T., Paunio, T., Pejovic-Milovancevic, M., Perkins, D. O., Pietiläinen, O., Pimm, J., Pocklington, A. J., Powell, J., Price, A., Pulver, A. E., Purcell, S. M., Quested, D., Rasmussen, H. B., Reichenberg, A., Reimers, M. a., Richards, A. L., Roffman, J. L., Roussos, P., Ruderfer, D. M., Salomaa, V., Sanders, A. R., Schall, U., Schubert, C. R., Schulze, T. G., Schwab, S. G., Scolnick, E. M., Scott, R. J., Seidman, L. J., Shi, J., Sigurdsson, E., Silagadze, T., Silverman, J. M., Sim, K., Slominsky, P., Smoller, J. W., So, H.-C., Spencer, C. a., Stahl, E. a., Stefansson, H., Steinberg, S., Stogmann, E., Straub, R. E., Strengman, E., Strohmaier, J., Scott Stroup, T., Subramaniam, M., Suvisaari, J., Svrakic, D. M., Szatkiewicz, J. P., Söderman, E., Thirumalai, S., Toncheva, D., Tosato, S., Veijola, J., Waddington, J., Walsh, D., Wang, D., Wang, Q., Webb, B. T., Weiser, M., Wildenauer, D. B., Williams, N. M., Williams, S., Witt, S. H., Wolen, A. R., Wong, E. H. M., Wormley, B. K., Simon Xi, H., Zai, C. C., Zheng, X., Zimprich, F., Wray, N. R., Stefansson, K., Visscher, P. M., Trust Case-Control Consortium, W., Adolfsson, R., Andreassen, O. a., Blackwood, D. H. R., Bramon, E., Buxbaum, J. D.,

Børglum, A. D., Cichon, S., Darvasi, A., Domenici, E., Ehrenreich, H., Esko, T., Gejman, P. V., Gill, M., Gurling, H., Hultman, C. M., Iwata, N., Jablensky, A. V., Jönsson, E. G., Kendler, K. S., Kirov, G., Knight, J., Lencz, T., Levinson, D. F., Li, Q. S., Liu, J., Malhotra, A. K., McCarroll, S. a., McQuillin, A., Moran, J. L., Mortensen, P. B., Mowry, B. J., Nöthen, M. M., Ophoff, R. a., Owen, M. J., Palotie, A., Pato, C. N., Petryshen, T. L., Posthuma, D., Rietschel, M., Riley, B. P., Rujescu, D., Sham, P. C., Sklar, P., St Clair, D., Weinberger, D. R., Wendland, J. R., Werge, T., Daly, M. J., Sullivan, P. F. and O'Donovan, M. C. (2014) 'Biological insights from 108 schizophrenia-associated genetic loci', *Nature*, 511, pp. 421-457.

Rippberger, H., van Gaalen, M. M., Schwarting, R. K. W. and Wohr, M. (2015) 'Environmental and Pharmacological Modulation of Amphetamine- Induced 50-kHz Ultrasonic Vocalizations in Rats.', *Current neuropharmacology*, 13(2), pp. 220–32.

Roussos, P., Giakoumaki, S. G., Georgakopoulos, A., Robakis, N. K. and Bitsios, P. (2011) 'The CACNA1C and ANK3 risk alleles impact on affective personality traits and startle reactivity but not on cognition or gating in healthy males', *Bipolar Disorders*, 13(3), pp. 250–259. doi: 10.1111/j.1399-5618.2011.00924.x.

Roussos, P., Mitchell, A. C., Voloudakis, G., Fullard, J. F., Pothula, V. M., Tsang, J., Stahl, E. A., Georgakopoulos, A., Ruderfer, D. M., Charney, A., Okada, Y., Siminovitch, K. A., Worthington, J., Padyukov, L., Klareskog, L., Gregersen, P. K., Plenge, R. M., Raychaudhuri, S., Fromer, M., Purcell, S. M., Brennand, K. J., Robakis, N. K., Schadt, E. E., Akbarian, S. and Sklar, P. (2014) 'A Role for Noncoding Variation in Schizophrenia', *Cell Reports*, 9(4), pp. 1417–1429. doi: 10.1016/j.celrep.2014.10.015.

Scardochio, T., Trujillo-Pisanty, I., Conover, K., Shizgal, P. and Clarke, P. B. S. (2015) 'The Effects of Electrical and Optical Stimulation of Midbrain Dopaminergic Neurons on Rat 50-kHz Ultrasonic Vocalizations', *Frontiers in Behavioral Neuroscience*, 9.

Schizophrenia Psychiatric Genome-Wide Association Study (GWAS) Consortium, T. S. P. G.-W. A. S. (GWAS) (2011) 'Genome-wide association study identifies five new schizophrenia loci.', *Nature genetics, 43,* pp. 969-976. doi: 10.1038/ng.940.

Schneider, M. and Koch, M. (2005) 'Deficient Social and Play Behavior in Juvenile and Adult Rats after Neonatal Cortical Lesion: Effects of Chronic Pubertal Cannabinoid Treatment', *Neuropsychopharmacology*, 30(5), pp. 944–957. doi: 10.1038/sj.npp.1300634.

Schultz, W. (2002) 'Getting formal with dopamine and reward', *Neuron*, 36(2) pp. 241–263. doi: 10.1016/S0896-6273(02)00967-4.

Schwarting, R. K. W., Jegan, N. and Wöhr, M. (2007) 'Situational factors, conditions and individual variables which can determine ultrasonic vocalizations in male adult Wistar rats', *Behavioural Brain Research*, 182(2), pp. 208–222. doi: 10.1016/j.bbr.2007.01.029.

Seffer, D., Rippberger, H., Schwarting, R. K. W. and Wöhr, M. (2015) 'Pro-social 50-kHz ultrasonic communication in rats: post-weaning but not post-adolescent social isolation leads to social impairments—phenotypic rescue by re-socialization', *Frontiers in Behavioral Neuroscience*, 9, pp. 102. doi: 10.3389/fnbeh.2015.00102.

Seffer, D., Schwarting, R. K. W. and Wöhr, M. (2014) 'Pro-social ultrasonic communication in rats: Insights from playback studies', *Journal of Neuroscience Methods*, 234, pp. 73–81. doi: 10.1016/j.jneumeth.2014.01.023.

Silverman, J. L., Yang, M., Lord, C. and Crawley, J. N. (2010) 'Behavioural phenotyping assays for mouse models of autism', *Nature Reviews Neuroscience*, 11(7), pp. 490–502. doi: 10.1038/nrn2851.

Simola, N., Fenu, S., Costa, G., Pinna, A., Plumitallo, A. and Morelli, M. (2012) 'Pharmacological characterization of 50-kHz ultrasonic vocalizations in rats: Comparison of the effects of different psychoactive drugs and relevance in drug-induced reward', *Neuropharmacology*, 63(2), pp. 224–234. doi: 10.1016/j.neuropharm.2012.03.013.

Sinnegger-Brauns, M. J., Huber, I. G., Koschak, A., Wild, C., Obermair, G. J., Einzinger, U., Hoda, J.-C., Sartori, S. B. and Striessnig, J. (2009) 'Expression and 1,4-dihydropyridine-binding properties of brain L-type calcium channel isoforms.', *Molecular pharmacology*, 75(2), pp. 407–14. doi: 10.1124/mol.108.049981.

Sittig, L. J., Carbonetto, P., Engel, K. A., Krauss, K. S., Barrios-Camacho, C. M. and Palmer, A. A. (2016) 'Genetic Background Limits Generalizability of Genotype-Phenotype Relationships', *Neuron*, 91(6), pp. 1253–1259. doi: 10.1016/j.neuron.2016.08.013.

Siviy, S. M. and Panksepp, J. (2011) 'In search of the neurobiological substrates for social playfulness in mammalian brains', *Neuroscience & Biobehavioral Reviews*, 35(9), pp. 1821–1830. doi: 10.1016/j.neubiorev.2011.03.006.

Sklar, P., Ripke, S., Scott, L. J., Andreassen, O. A., Cichon, S., Craddock, N., Edenberg, H. J., Nurnberger, J. I., Rietschel, M., Blackwood, D., Corvin, A., Flickinger, M., Guan, W., Mattingsdal, M., McQuillin, A., Kwan, P., Wienker, T. F., Daly, M., Dudbridge, F., Holmans, P. A., Lin, D., Burmeister, M., Greenwood, T. A., Hamshere, M. L., Muglia, P., Smith, E. N., Zandi, P. P., Nievergelt, C. M., McKinney, R., Shilling, P. D., Schork, N. J., Bloss, C. S., Foroud, T., Koller, D. L., Gershon, E. S., Liu, C., Badner, J. A., Scheftner, W. A., Lawson, W. B., Nwulia, E. A., Hipolito, M., Coryell, W., Rice, J., Byerley, W., McMahon, F. J., Schulze, T. G., Berrettini, W., Lohoff, F. W., Potash, J. B., Mahon, P. B., McInnis, M. G., Zöllner, S., Zhang, P., Craig, D. W., Szelinger, S., Barrett, T. B., Breuer, R., Meier, S., Strohmaier, J., Witt, S. H., Tozzi, F., Farmer, A., McGuffin, P., Strauss, J., Xu, W., Kennedy, J. L., Vincent, J. B., Matthews, K., Day, R., Ferreira, M. a, O'Dushlaine, C., Perlis, R., Raychaudhuri, S., Ruderfer, D., Hyoun, P. L., Smoller, J. W., Li, J., Absher, D., Thompson, R. C., Meng, F. G., Schatzberg, A. F., Bunney, W. E., Barchas, J. D., Jones, E. G., Watson, S. J., Myers, R. M., Akil, H., Boehnke, M., Chambert, K., Moran, J., Scolnick, E., Djurovic, S., Melle, I., Morken, G., Gill, M., Morris, D., Quinn, E., Mühleisen, T. W., Degenhardt, F. A., Mattheisen, M., Schumacher, J., Maier, W., Steffens, M., Propping, P., Nöthen, M. M., Anjorin, A., Bass, N., Gurling, H., Kandaswamy, R., Lawrence, J., McGhee, K., McIntosh, A., McLean, A. W., Muir, W. J., Pickard, B. S., Breen, G., St. Clair, D., Caesar, S., Gordon-Smith, K., Jones, L., Fraser, C., Green, E. K., Grozeva, D., Jones, I. R., Kirov, G., Moskvina, V., Nikolov, I., O'Donovan, M. C., Owen, M. J., Collier, D. a, Elkin, A., Williamson, R., Young, A. H., Ferrier, I. N., Stefansson, K., Stefansson, H., Þorgeirsson, Þ., Steinberg, S., Gustafsson, Ó., Bergen, S. E., Nimgaonkar, V., Hultman, C., Landén, M., Lichtenstein, P., Sullivan, P. F., Schalling, M., Osby, U., Backlund, L., Frisén, L., Langstrom, N., Jamain, S., Leboyer, M., Etain, B., Bellivier, F., Petursson, H., Sigurðsson, E., Müller-Mysok, B., Lucae, S., Schwarz,

M., Schofield, P. R., Martin, N., Montgomery, G. W., Lathrop, M., Óskarsson, H., Bauer, M., Wright, A., Mitchell, P. B., Hautzinger, M., Reif, A., Kelsoe, J. R. and Purcell, S. M. (2011) 'Large-scale genome-wide association analysis of bipolar disorder identifies a new susceptibility locus near ODZ4', *Nature Genetics*, 43(10), pp. 977–983. doi: 10.1038/ng.943.

Sklar, P., Smoller, J. W., Fan, J., Ferreira, M. A. R., Perlis, R. H., Chambert, K., Nimgaonkar, V. L., McQueen, M. B., Faraone, S. V, Kirby, A., de Bakker, P. I. W., Ogdie, M. N., Thase, M. E., Sachs, G. S., Todd-Brown, K., Gabriel, S. B., Sougnez, C., Gates, C., Blumenstiel, B., Defelice, M., Ardlie, K. G., Franklin, J., Muir, W. J., McGhee, K. A., MacIntyre, D. J., McLean, A., VanBeck, M., McQuillin, A., Bass, N. J., Robinson, M., Lawrence, J., Anjorin, A., Curtis, D., Scolnick, E. M., Daly, M. J., Blackwood, D. H., Gurling, H. M. and Purcell, S. M. (2008) 'Whole-genome association study of bipolar disorder', *Molecular Psychiatry*, 13(6), pp. 558–569. doi: 10.1038/sj.mp.4002151.

Soeiro-de-Souza, M. G., Bio, D. S., Dias, V. V., Vieta, E., Machado-Vieira, R. and Moreno, R. A. (2013) 'The CACNA1C risk allele selectively impacts on executive function in bipolar type I disorder', *Acta Psychiatrica Scandinavica*. doi: 10.1111/acps.12073.

Splawski, I., Timothy, K. W., Decher, N., Kumar, P., Sachse, F. B., Beggs, A. H., Sanguinetti, M. C. and Keating, M. T. (2005) 'Severe arrhythmia disorder caused by cardiac L-type calcium channel mutations.', *Proceedings of the National Academy of Sciences of the United States of America, 10(23),* pp. 8089-8096. doi: 10.1073/pnas.0502506102.

Splawski, I., Timothy, K. W., Sharpe, L. M., Decher, N., Kumar, P., Bloise, R., Napolitano, C., Schwartz, P. J., Joseph, R. M., Condouris, K., Tager-Flusberg, H., Priori, S. G., Sanguinetti, M. C. and Keating, M. T. (2004) '$Ca_V1.2$ Calcium Channel Dysfunction Causes a Multisystem Disorder Including Arrhythmia and Autism', *Cell*, 119(1), pp. 19–31. doi: 10.1016/j.cell.2004.09.011.

Starnawska, A., Demontis, D., Pen, A., Hedemand, A., Nielsen, A. L., Staunstrup, N. H., Grove, J., Als, T. D., Jarram, A., O'Brien, N. L., Mors, O., McQuillin, A., Børglum, A. D. and Nyegaard, M. (2016) 'CACNA1C hypermethylation is associated with bipolar disorder', *Translational Psychiatry*, 6(6), pp. e831. doi: 10.1038/tp.2016.99.

Strohmaier, J., Amelang, M., Hothorn, L. A., Witt, S. H., Nieratschker, V., Gerhard, D., Meier, S., Wüst, S., Frank, J., Loerbroks, A., Rietschel, M., Stürmer, T. and Schulze, T. G. (2013) 'The psychiatric vulnerability gene CACNA1C and its sex-specific relationship with personality traits, resilience factors and depressive symptoms in the general population.', *Molecular psychiatry*, 18(5), pp. 607–13. doi: 10.1038/mp.2012.53.

Sun, W., Poschmann, J., Cruz-Herrera del Rosario, R., Parikshak, N. N., Hajan, H. S., Kumar, V., Ramasamy, R., Belgard, T. G., Elanggovan, B., Wong, C. C. Y., Mill, J., Geschwind, D. H. and Prabhakar, S. (2016) 'Histone Acetylome-wide Association Study of Autism Spectrum Disorder', *Cell*, 167(5), p. 1385–1397.e11. doi: 10.1016/j.cell.2016.10.031.

Terrillion, C. E., Dao, D. T., Cachope, R., Lobo, M. K., Puche, A. C., Cheer, J. F. and Gould, T. D. (2017) 'Reduced levels of Cacna1c attenuate mesolimbic dopamine system function', *Genes, Brain and Behavior*, 16(5), pp. 495–505. doi: 10.1111/gbb.12371.

Terrillion, C. E., Francis, T. C., Puche, A. C., Lobo, M. K. and Gould, T. D. (2017) 'Decreased Nucleus Accumbens Expression of Psychiatric Disorder Risk Gene Cacna1c

Promotes Susceptibility to Social Stress', *International Journal of Neuropsychopharmacology*, 20(5), pp. 428–433. doi: 10.1093/ijnp/pyw112.

Tesli, M., Skatun, K. C., Ousdal, O. T., Brown, A. A., Thoresen, C., Agartz, I., Melle, I., Djurovic, S., Jensen, J. and Andreassen, O. A. (2013) 'CACNA1C Risk Variant and Amygdala Activity in Bipolar Disorder, Schizophrenia and Healthy Controls', *PLOS ONE*, 8(2), p. e56970.

Thompson, B., Leonard, K. C. and Brudzynski, S. M. (2006) 'Amphetamine-induced 50 kHz calls from rat nucleus accumbens: A quantitative mapping study and acoustic analysis', *Behavioural Brain Research*, 168(1), pp. 64–73. doi: 10.1016/j.bbr.2005.10.012.

Thor, D. H. and Holloway, W. R. (1983) 'Play-solicitation behavior in juvenile male and female rats', *Animal Learning & Behavior*, 11(2), pp. 173–178.

Thor, D. H. and Holloway, W. R. (1984) 'Social play in juvenile rats: A decade of methodological and experimental research', *Neuroscience and Biobehavioral Reviews, 8(4),* pp.455-464. doi: 10.1016/0149-7634(84)90004-6.

Trezza, V., Baarendse, P. J. J. and Vanderschuren, L. J. (2010) 'The pleasures of play: pharmacological insights into social reward mechanisms.', *Trends in pharmacological sciences*, 31(10), pp. 463–9. doi: 10.1016/j.tips.2010.06.008.

Vanderschuren, L. J., Achterberg, E. J. M. and Trezza, V. (2016) 'The neurobiology of social play and its rewarding value in rats', *Neuroscience and Biobehavioral Reviews*, 70, pp. 86–105. doi: 10.1016/j.neubiorev.2016.07.025.

Vanderschuren, L. J., Stein, E. A., Wiegant, V. M. and Van Ree, J. M. (1995) 'Social isolation and social interaction alter regional brain opioid receptor binding in rats', *European Neuropsychopharmacology*, 5(2), pp. 119–127. doi: 10.1016/0924-977X(95)00010-M.

Ward, I. L. and Stehm, K. E. (1991) 'Prenatal stress feminizes juvenile play patterns in male rats', *Physiology & Behavior*, 50(3), pp. 601–605.

Webber, E. S., Harmon, K. M., Beckwith, T. J., Peña, S., Burgdorf, J., Panksepp, J. and Cromwell, H. C. (2012) 'Selective breeding for 50kHz ultrasonic vocalization emission produces alterations in the ontogeny and regulation of rough-and-tumble play', *Behavioural Brain Research*, 229(1), pp. 138–144. doi: 10.1016/j.bbr.2012.01.012.

Wessa, M., Linke, J., Witt, S. H., Nieratschker, V., Esslinger, C., Kirsch, P., Grimm, O., Hennerici, M. G., Gass, A., King, A. V. and Rietschel, M. (2010) 'The CACNA1C risk variant for bipolar disorder influences limbic activity', *Molecular Psychiatry*, 15, pp. 1126-1127. doi: 10.1038/mp.2009.103.

Willadsen, M., Seffer, D., Schwarting, R. K. W. and Wöhr, M. (2014) 'Rodent ultrasonic communication: Male prosocial 50-kHz ultrasonic vocalizations elicit social approach behavior in female rats (Rattus norvegicus).', *Journal of comparative psychology,* 128(1), pp. 56–64. doi: 10.1037/a0034778.

Willuhn, I., Tose, A., Wanat, M. J., Hart, A. S., Hollon, N. G., Phillips, P. E. M., Schwarting, R. K. W. and Wöhr, M. (2014) 'Phasic Dopamine Release in the Nucleus Accumbens in Response to Pro-Social 50 kHz Ultrasonic Vocalizations in Rats', *The Journal of Neuroscience*, 34(32), pp. 10616-10623. doi: 10.1523/JNEUROSCI.1060-14.2014.

Wise, R. A. (2004) 'Dopamine, learning and motivation', *Nature Reviews Neuroscince*, 5(6), pp. 483–494.

Wöhr, M., Houx, B., Schwarting, R. K. W. and Spruijt, B. (2008) 'Effects of experience and context on 50-kHz vocalizations in rats', *Physiology and Behavior*, 93(4–5), pp. 766–776. doi: 10.1016/j.physbeh.2007.11.031.

Wöhr, M., Rippberger, H., Schwarting, R. K. W. and Van Gaalen, M. M. (2015) 'Critical involvement of 5-HT2C receptor function in amphetamine-induced 50-kHz ultrasonic vocalizations in rats', *Psychopharmacology*, 232(10), pp. 1817–1829. doi: 10.1007/s00213-014-3814-9.

Wöhr, M. and Scattoni, M. L. (2013) 'Behavioural methods used in rodent models of autism spectrum disorders: Current standards and new developments', *Behavioural Brain Research*, 251, pp. 5–17. doi: 10.1016/j.bbr.2013.05.047.

Wöhr, M. and Schwarting, R. K. W. (2007) 'Ultrasonic communication in rats: Can playback of 50-kHz calls induce approach behavior?', *PLoS ONE*, 2(12).

Wöhr, M. and Schwarting, R. K. W. (2009) 'Ultrasonic communication in rats: Effects of morphine and naloxone on vocal and behavioral responses to playback of 50-kHz vocalizations', *Pharmacology Biochemistry and Behavior*, 94(2), pp. 285–295.

Wöhr, M. and Schwarting, R. K. W. (2012) 'Testing social acoustic memory in rats: Effects of stimulus configuration and long-term memory on the induction of social approach behavior by appetitive 50-kHz ultrasonic vocalizations', *Neurobiology of Learning and Memory*, 98(2), pp. 154–164. doi: 10.1016/j.nlm.2012.05.004.

Wöhr, M. and Schwarting, R. K. W. (2013) 'Affective communication in rodents: Ultrasonic vocalizations as a tool for research on emotion and motivation', *Cell and Tissue Research*, 354(1), pp. 81–97. doi: 10.1007/s00441-013-1607-9.

Wray, N. R., Pergadia, M. L., Blackwood, D. H. R., Penninx, B. W. J. H., Gordon, S. D., Nyholt, D. R., Ripke, S., MacIntyre, D. J., McGhee, K. A., Maclean, A. W., Smit, J. H., Hottenga, J. J., Willemsen, G., Middeldorp, C. M., de Geus, E. J. C., Lewis, C. M., McGuffin, P., Hickie, I. B., van den Oord, E. J. C. G., Liu, J. Z., Macgregor, S., McEvoy, B. P., Byrne, E. M., Medland, S. E., Statham, D. J., Henders, A. K., Heath, A. C., Montgomery, G. W., Martin, N. G., Boomsma, D. I., Madden, P. A. F. and Sullivan, P. F. (2012) 'Genome-wide association study of major depressive disorder: new results, meta-analysis, and lessons learned.', *Molecular psychiatry,* 17, pp. 36-48. doi: 10.1038/mp.2010.109.

Wright, J. M., Deng, L. and Clarke, P. B. S. (2012) 'Failure of rewarding and locomotor stimulant doses of morphine to promote adult rat 50-kHz ultrasonic vocalizations', *Psychopharmacology*, 224(4), pp. 477–487. doi: 10.1007/s00213-012-2776-z.

Wright, J. M., Gourdon, J. C. and Clarke, P. B. S. (2010) 'Identification of multiple call categories within the rich repertoire of adult rat 50-kHz ultrasonic vocalizations: effects of amphetamine and social context', *Psychopharmacology*, 211(1), pp. 1–13. doi: 10.1007/s00213-010-1859-y.

Yang, M. and Crawley, J. N. (2009) 'Simple behavioral assessment of mouse olfaction', *Current Protocols in Neuroscience, 48(1),* pp. 8.24.1-8.24.12.

Zamponi, G. W. (2016) ‘Targeting voltage-gated calcium channels in neurological and psychiatric diseases.’, *Nature reviews. Drug discovery*, 15(1), pp. 19–34. doi: 10.1038/nrd.2015.5.

Zuccotti, A., Clementi, S., Reinbothe, T., Torrente, A., Vandael, D. H. and Pirone, A. (2011) ‘Structural and functional differences between L-type calcium channels: Crucial issues for future selective targeting’, *Trends in Pharmacological Sciences*, *32(6),* pp. 366-375. doi: 10.1016/j.tips.2011.02.012.

FIGURE LEGENDS

Figure 1: $Ca_V1.2$ protein levels in *Cacna1c*$^{+/-}$ rats and *Cacna1c*$^{+/+}$ littermate controls. $Ca_V1.2$ expression levels were analyzed by Western blot from cortical tissue of female *Cacna1c*$^{+/-}$ rats (white bars; N=6) and *Cacna1c*$^{+/+}$ littermate controls (black bars; N=6). The bar graphs (left panel) were obtained by densitometric quantification of the Western blot data. The results are expressed as percentage of *Cacna1c*$^{+/+}$ littermate control values after normalization to the loading control vinculin. The $Ca_V1.2$ level of *Cacna1c*$^{+/+}$ littermate controls is set as 100 %. The immunoblots (right panel) show one representative example per genotype. Data are presented as mean±SEM. * $p<0.050$ vs. *Cacna1c*$^{+/+}$ littermate controls.

Figure 2: Rough-and-tumble play behavior and concomitant 50-kHz USV emission in *Cacna1c*$^{+/-}$ rats and *Cacna1c*$^{+/+}$ littermate controls. (**A**) Time spent playing; (**B**) time spent pinning; and (**C**) 50-kHz USV emission across the three play sessions in female *Cacna1c*$^{+/-}$ rats (white circles; N=10) and *Cacna1c*$^{+/+}$ littermate controls (black circles; N=10). (**D**) Representative, composite, and consolidated ethograms of a *Cacna1c*$^{+/-}$ rat pair (upper panels) and a *Cacna1c*$^{+/+}$ littermate control pair (lower panels) of the first and third play session, respectively. Pinning (blue), wrestling (green), and chasing (brown) events are depicted, together with 50-kHz USV (red) for the entire 5 min play sessions. Data are presented as mean±SEM. $^{\#}$ $p<0.050$ vs. first play session; * $p<0.050$ vs. *Cacna1c*$^{+/+}$ littermate controls.

Figure 3: Social approach behavior evoked by pro-social 50-kHz USV playback in *Cacna1c*$^{+/-}$ rats and *Cacna1c*$^{+/+}$ littermate controls. (**A**) Exemplary spectrograms of acoustic stimuli used for playback, namely pro-social 50-kHz USV (upper panel) and time- and amplitude-matched White Noise (lower panel). (**B**) Schematic illustration of the radial maze used for playback depicting proximal (black), distal (grey), and neutral (white) arms relative to the active ultrasonic loudspeaker. (**C**) Change in locomotor activity in female *Cacna1c*$^{+/-}$ rats (white bars; N=20) and *Cacna1c*$^{+/+}$ littermate controls (black bars; N=20) as measured by total arm entries per minute during (left) and after (right) 50-kHz USV and White

Noise playback, as compared to the 5 minutes baseline period before playback. (**D**) Change in social approach behavior in female *Cacna1c*$^{+/-}$ rats (white bars; N=20) and *Cacna1c*$^{+/+}$ littermate controls (black bars; N=20) as measured by time spent on proximal (PROX) and distal (DIST) arms per minute during (left) and after (right) 50-kHz USV playback, as compared to the 5 minutes baseline period before playback. (E) Change in avoidance behavior in female *Cacna1c*$^{+/-}$ rats (white bars; N=20) and *Cacna1c*$^{+/+}$ littermate controls (black bars; N=20) as measured by time spent on proximal (PROX) and distal (DIST) arms per minute during (left) and after (right) White Noise playback, as compared to the 5 minutes baseline period before playback. The dashed line represents baseline levels. Data are presented as mean±SEM. $^{\#}$ p<0.050 vs. baseline levels; ^{x} p<0.050 vs. distal.

Figure 4: Response calls evoked by pro-social 50-kHz USV playback in *Cacna1c*$^{+/-}$ rats and *Cacna1c*$^{+/+}$ littermate controls. Emission of 22-kHz USV in (**A**) female *Cacna1c*$^{+/-}$ rats and (**B**) *Cacna1c*$^{+/+}$ littermate controls, with individual 22-kHz USV (red) per individual rat. (**C**) Total number of 22-kHz USV in *Cacna1c*$^{+/-}$ females (grey area; N=20) and *Cacna1c*$^{+/+}$ littermate controls (black area; N=20). Emission of 22-kHz USV in (**D**) male *Cacna1c*$^{+/-}$ rats and (**E**) *Cacna1c*$^{+/+}$ littermate controls, with individual 22-kHz USV (red) per individual rat. (**F**) Total number of 22-kHz USV in *Cacna1c*$^{+/-}$ males (grey area; N=20) and *Cacna1c*$^{+/+}$ littermate controls (black area; N=20). The dashed lines represent beginning and end of 50-kHz USV playback.

FIGURE 1

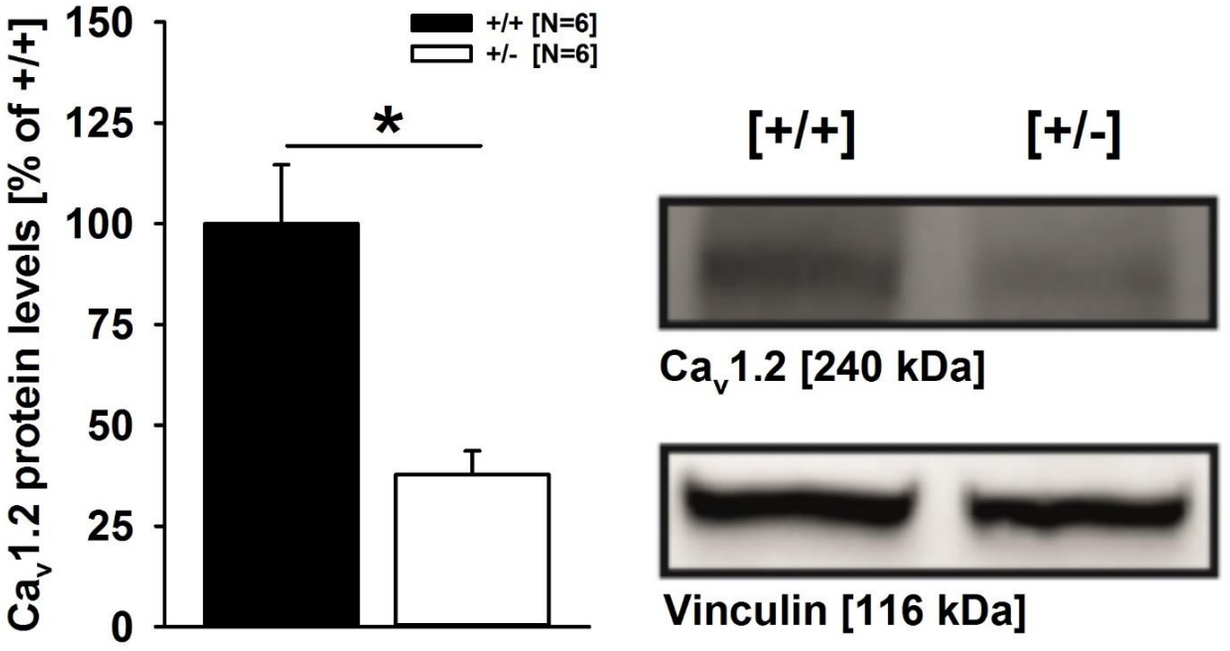

+/+ [N=6]
+/- [N=6]
*
$Ca_v1.2$ protein levels [% of +/+]
150
125
100
75
50
25
0
[+/+]
[+/-]
$Ca_v1.2$ [240 kDa]
Vinculin [116 kDa]

FIGURE 2

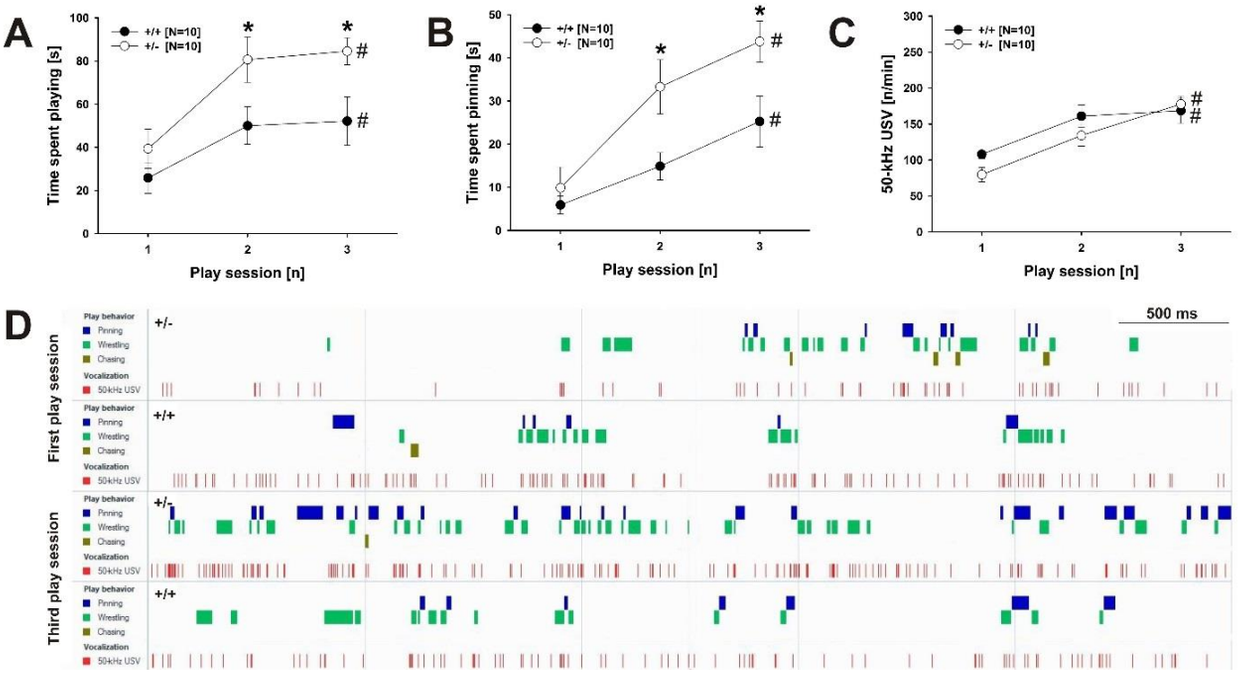

A
Time spent playing [s]
+/+ [N=10]
+/- [N=10]
Play session [n]
B
Time spent pinning [s]
+/+ [N=10]
+/- [N=10]
Play session [n]
C
50-kHz USV [n/min]
+/+ [N=10]
+/- [N=10]
Play session [n]
D
500 ms
First play session
Third play session
Play behavior
Pinning
Wrestling
Chasing
Vocalization
50-kHz USV
+/-
+/+

FIGURE 3

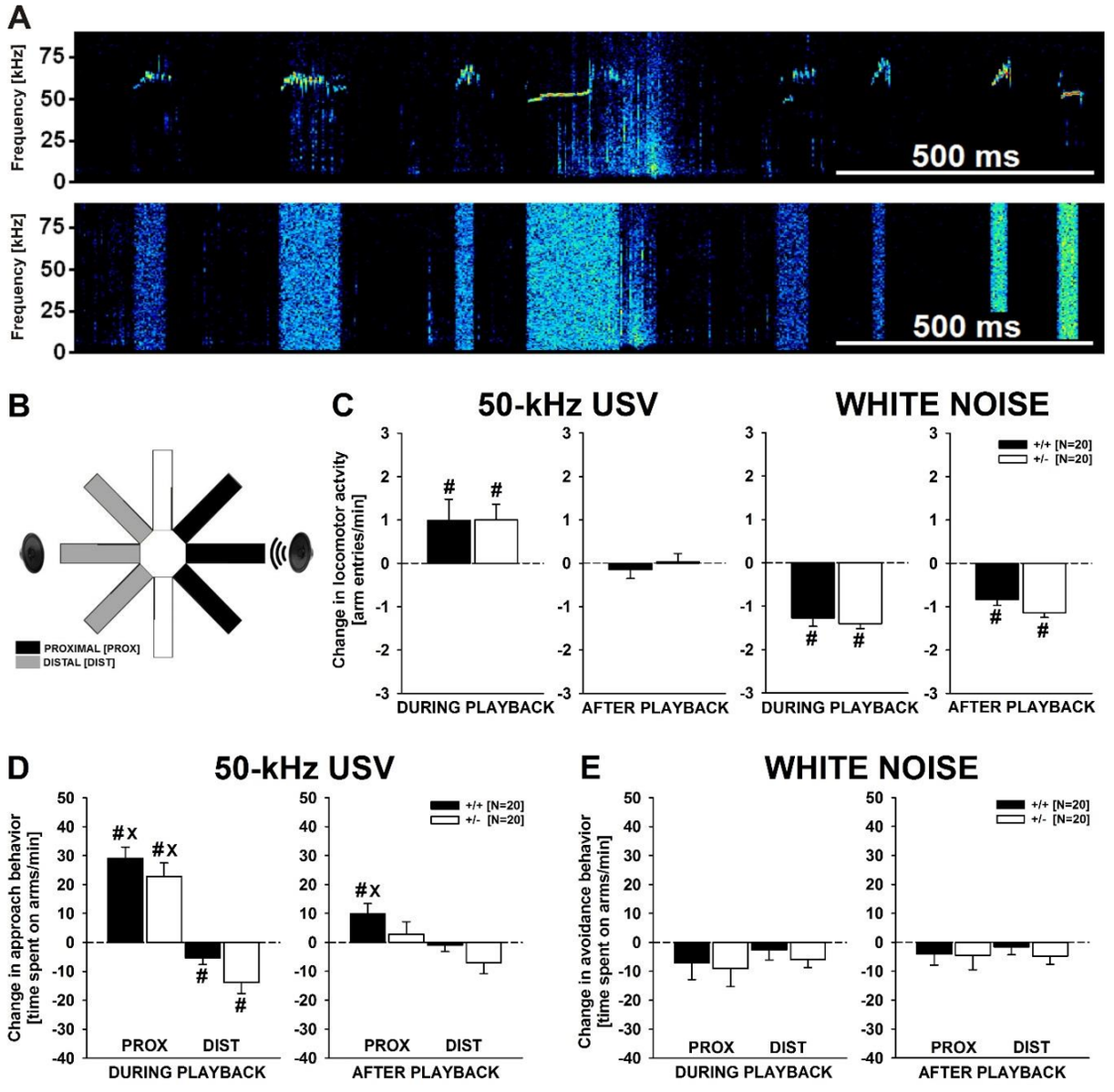
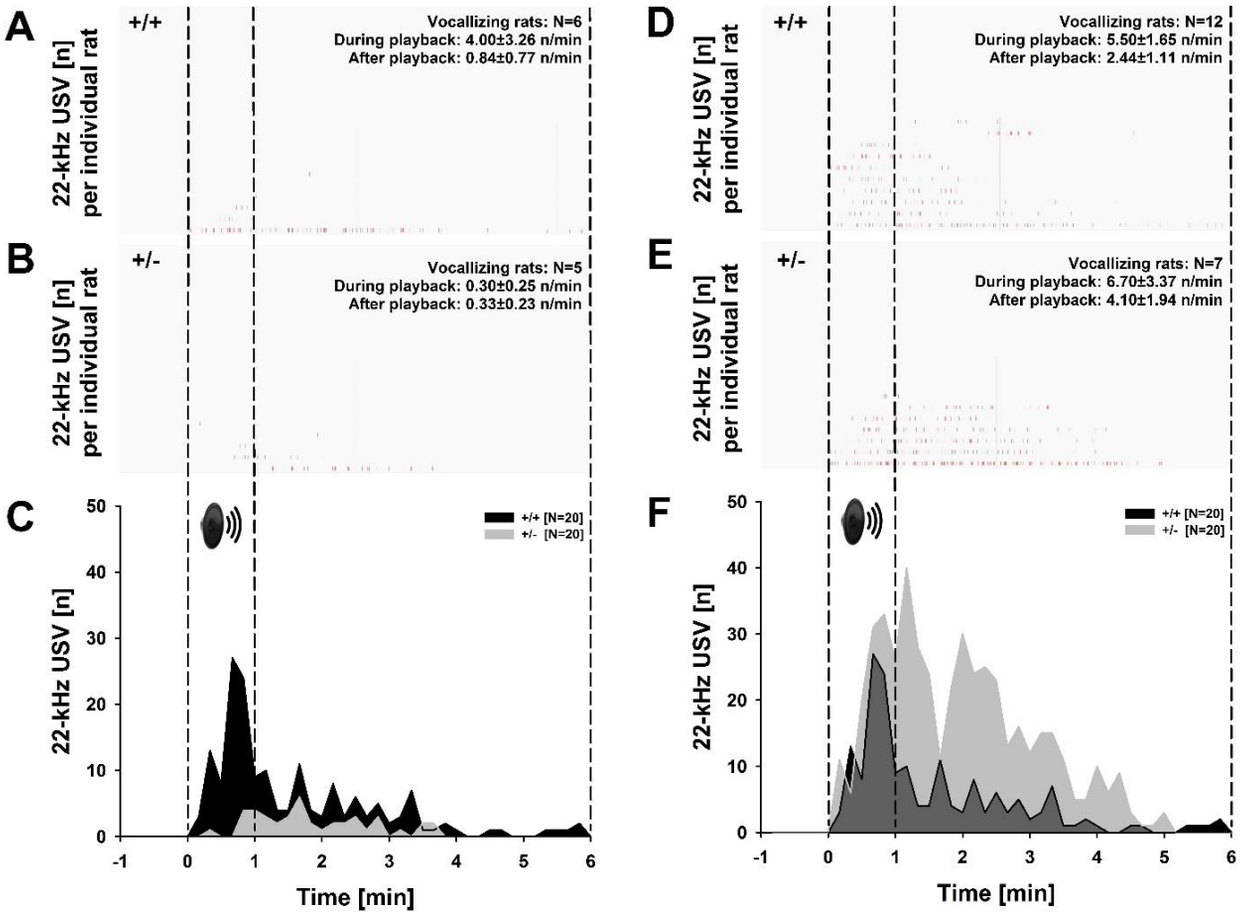

A
Frequency [kHz]
75
50
25
0
500 ms
B
PROXIMAL [PROX]
DISTAL [DIST]
C
50-kHz USV
WHITE NOISE
Change in locomotor actvity [arm entries/min]
+/+ [N=20]
+/- [N=20]
DURING PLAYBACK
AFTER PLAYBACK
D
50-kHz USV
Change in approach behavior [time spent on arms/min]
PROX
DIST
E
WHITE NOISE
Change in avoidance behavior [time spent on arms/min]

FIGURE 4

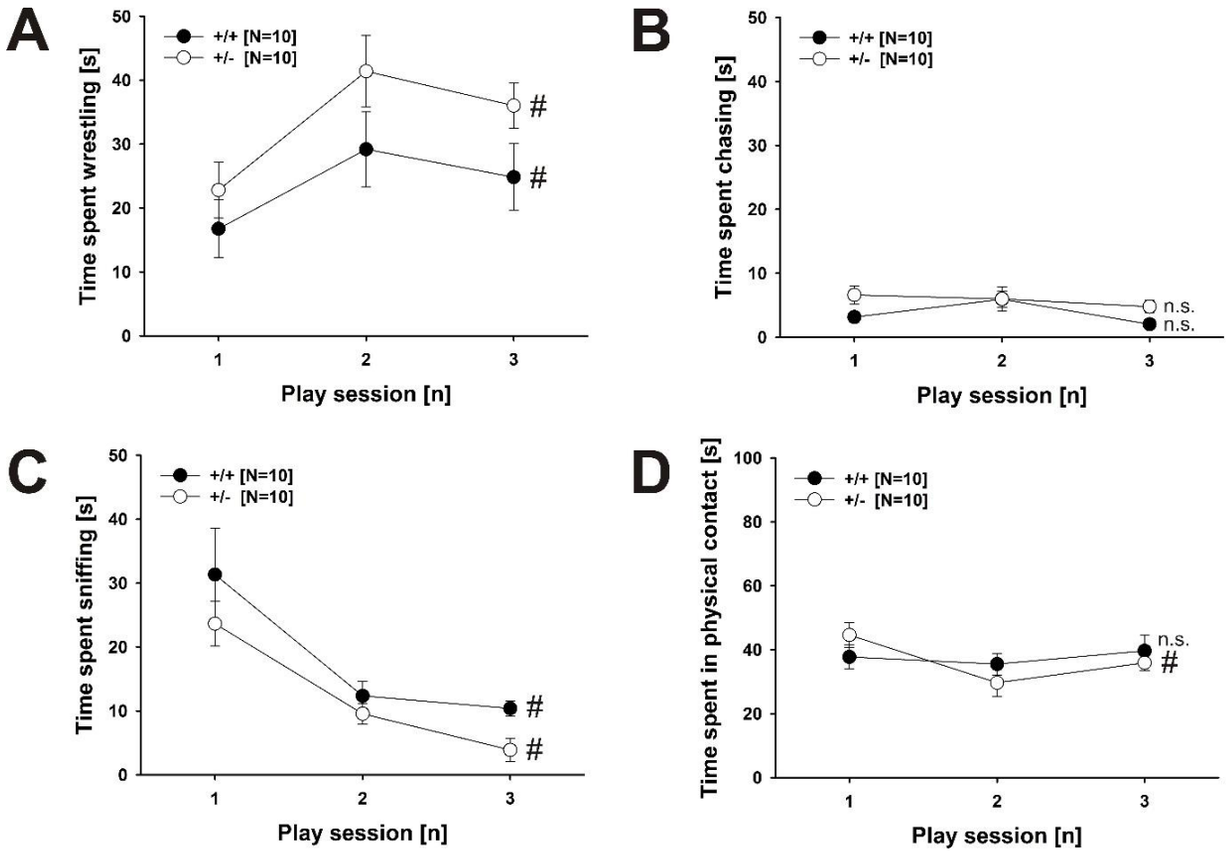

A
+/+
22-kHz USV [n] per individual rat
Vocallizing rats: N=6
During playback: 4.00±3.26 n/min
After playback: 0.84±0.77 n/min
B
+/-
22-kHz USV [n] per individual rat
Vocallizing rats: N=5
During playback: 0.30±0.25 n/min
After playback: 0.33±0.23 n/min
C
22-kHz USV [n]
+/+ [N=20]
+/- [N=20]
Time [min]
D
+/+
22-kHz USV [n] per individual rat
Vocallizing rats: N=12
During playback: 5.50±1.65 n/min
After playback: 2.44±1.11 n/min
E
+/-
22-kHz USV [n] per individual rat
Vocallizing rats: N=7
During playback: 6.70±3.37 n/min
After playback: 4.10±1.94 n/min
F
22-kHz USV [n]
+/+ [N=20]
+/- [N=20]
Time [min]

Supplementary Materials

Title: Sex-dependent effects of *Cacna1c* haploinsufficiency on juvenile social play behavior and pro-social 50-kHz ultrasonic communication in rats

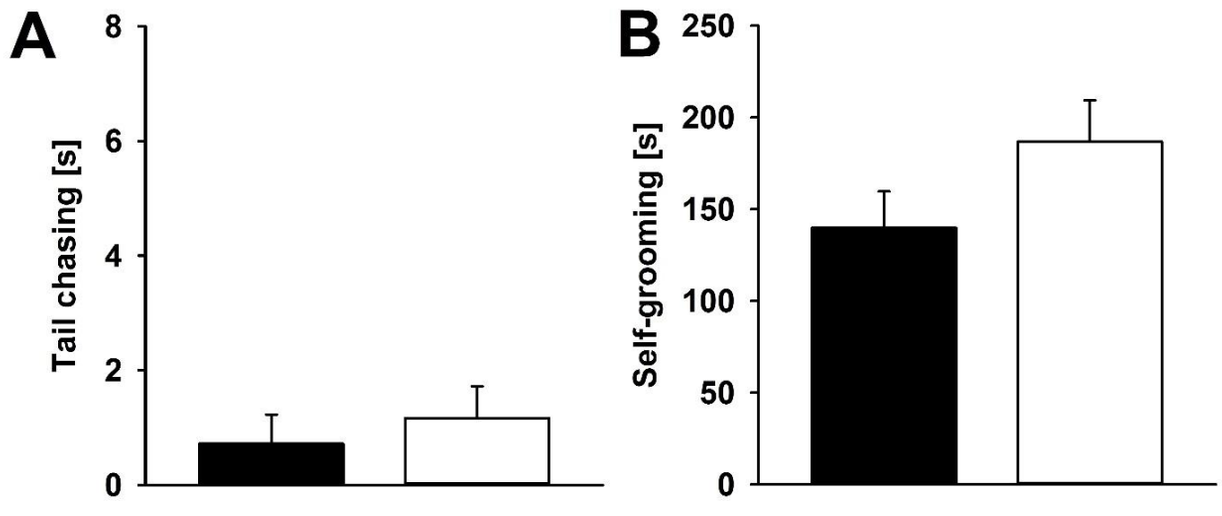
Authors: Theresa M. Kisko, Moria D. Braun, Susanne Michels, Stephanie H. Witt, Marcella Rietschel, Carsten Culmsee, Rainer K.W. Schwarting, Markus Wöhr

SUPPLEMENTARY FIGURE S1



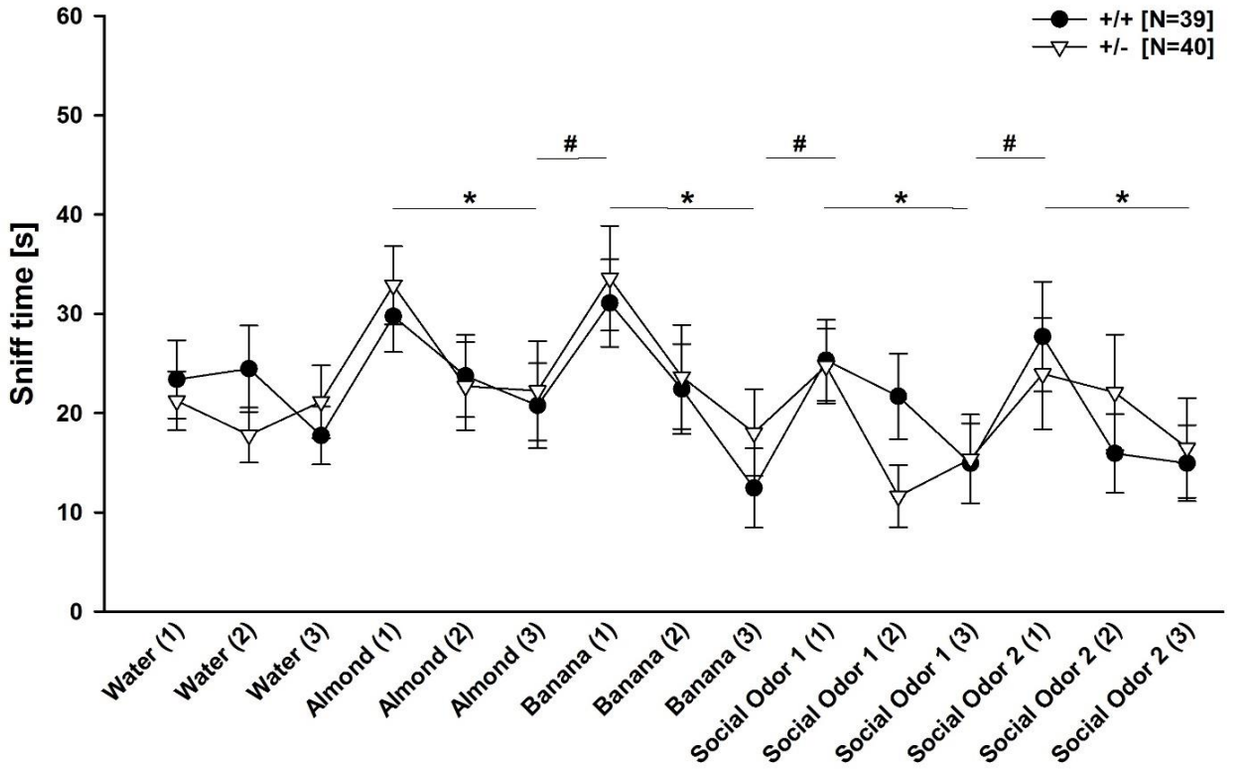
Supplementary Figure S1: Rough-and-tumble play behavior and non-play social behavior in *Cacna1c*$^{+/-}$ rats and *Cacna1c*$^{+/+}$ littermate controls. (**A**) Time spent wrestling; (**B**) time spent chasing; (**C**) time spent sniffing; and (**D**) time spent in physical contact across the three play sessions in female *Cacna1c*$^{+/-}$ rats (white circles; N=10) and *Cacna1c*$^{+/+}$ littermate controls (black circles; N=10). Data are presented as mean±SEM. $^{\#}$ $p<0.050$ vs. first play session.

SUPPLEMENTARY FIGURE S2



Supplementary Figure S2: Repetitive and stereotyped patterns of behavior in *Cacna1c*$^{+/-}$ rats and *Cacna1c*$^{+/+}$ littermate controls. (**A**) Time spent tail chasing and (**B**) self-grooming in female *Cacna1c*$^{+/-}$ rats (white bars; N=20) and *Cacna1c*$^{+/+}$ littermate controls (black bars; N=20). Data are presented as mean±SEM.

SUPPLEMENTARY FIGURE S2



Supplementary Figure S3: Olfactory habituation and dishabituation in *Cacna1c*$^{+/-}$ rats and *Cacna1c*$^{+/+}$ littermate controls. Time spent sniffing odor stimuli in *Cacna1c*$^{+/-}$ rats (white triangles; N=40) and *Cacna1c*$^{+/+}$ littermate controls (black circles; N=39). Data are presented as mean±SEM. $^{\#}$ $p<0.050$ vs. third stimulus exposure; * $p<0.050$ vs. first stimulus exposure.

TABLE 1

Body Weight	Females		Males	
Behavioral Paradigm	*Cacna1c* $^{+/+}$	*Cacna1c* $^{+/-}$	*Cacna1c* $^{+/+}$	*Cacna1c* $^{+/-}$
50-kHz USV playback; PND 24±3	62.55±1.85 g	51.37±1.88 g*	63.75±2.95 g	60.10±3.05 g
Rough-and-tumble play; PND 32-34	96.25±2.38 g	86.55±2.02 g*	105.80±3.69 g	103.95±3.84 g
Repetitive behavior; PND 64±3	219.45±3.57 g	213.75±4.03 g	337.25±5.80 g	338.65±6.12 g

Notes: USV = Ultrasonic vocalizations; PND = Postnatal day; * p<0.050 vs. *Cacna1c* $^{+/+}$ littermate controls; Male data were reported before (Kisko et al., in press) and included for the sake of comparison

Study III

Title: Sex-specific effects of *Cacna1c* haploinsufficiency on object recognition, spatial memory, and reversal learning capabilities in rats

Authors: Moria D. Braun, Theresa M. Kisko, Débora Dalla Vecchia, Roberto Andreatini, Rainer K.W. Schwarting, Markus Wöhr

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Sex-specific effects of *Cacna1c* haploinsufficiency on object recognition, spatial memory, and reversal learning capabilities in rats

Cacna1c and memory

Original Research Article

Moria D. Braun*[1], Theresa M. Kisko*[1], Débora Dalla Vecchia[2], Roberto Andreatini[2], Rainer K.W. Schwarting[1,3], Markus Wöhr[1,3]

[1] Behavioral Neuroscience, Experimental and Biological Psychology, Philipps-University of Marburg, Gutenberg-Str. 18, D-35032 Marburg, Germany

[2] Laboratory of Physiology and Pharmacology of the Central Nervous System, Department of Pharmacology, Federal University of Paraná, Centro Politécnico, 81540-990 Curitiba, PR, Brazil

[3] Center for Mind, Brain and Behavior, Philipps-University of Marburg, Hans-Meerwein-Str. 6, D-35032 Marburg, Germany

* shared first authorship

Correspondence should be addressed to:

Markus Wöhr
Behavioral Neuroscience
Experimental and Biological Psychology
Philipps-University of Marburg
Gutenbergstr. 18, 35032 Marburg, Germany
Fax: +6421 28 23610, Tel: +6421 28 23612
E-mail: markus.woehr@staff.uni-marburg.de

ABSTRACT

The *CACNA1C* gene is strongly implicated in the etiology of multiple major neuropsychiatric disorders, such as bipolar disorder, major depression, and schizophrenia, with cognitive deficits being a common feature. It is unclear, however, by which mechanisms *CACNA1C* variants advance the risk of developing neuropsychiatric disorders. This study set out to investigate cognitive functioning in a newly developed genetic *Cacna1c* rat model. Specifically, spatial and reversal learning, as well as object recognition memory were assessed in heterozygous *Cacna1c*$^{+/-}$ rats and compared to wildtype *Cacna1c*$^{+/+}$ littermate controls in both sexes. Our results show that both *Cacna1c*$^{+/+}$ and *Cacna1c*$^{+/-}$ animals were able to learn the rewarded arm configuration of a radial maze over the course of 7 days. Both groups also showed reversal learning patterns indicative of intact abilities. In females, genotype differences were evident in the initial spatial learning phase, with *Cacna1c*$^{+/-}$ females showing hypo-activity and fewer mixed errors. In males, a difference was found during probe trials for both learning phases, with *Cacna1c*$^{+/-}$ rats displaying better distinction between previously baited and non-baited arms; and regarding cognitive flexibility in favor of the *Cacna1c*$^{+/+}$ animals. All experimental groups proved to be sensitive to reward magnitude and fully able to distinguish between novel and familiar objects in the novel object recognition task. Taken together, these results indicate that *Cacna1c* haploinsufficiency has a minor, but positive impact on (spatial) memory functions in rats.

Key Words: Cav1.2, Calcium, Cognitive flexibility, Reward sensitivity, Affective disorder

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INTRODUCTION

The *CACNA1C* gene is strongly implicated in the etiology of multiple major neuropsychiatric disorders. *CACNA1C* variants, such as the single nucleotide polymorphism (SNP) rs1006737, are among the best replicated findings from genome-wide association studies (GWAS) and clinical investigations aiming at the identification of genetic risk factors in psychiatry. Relevant neuropsychiatric disorders include bipolar disorder (BPD; Ferreira et al., 2008; Sklar et al., 2008), major depression (MDD; Green et al., 2010; Dao et al., 2010), and schizophrenia (SCZ; Nyegaard et al., 2010, Ripke et al., 2014; for review see Kabir et al., 2016), with a lifetime prevalence ranging from 3.5% in psychotic disorders (Perälä et al., 2007) to 21% in affective disorders (Kessler et al., 2005). *CACNA1C* encodes the pore-forming alpha-1C subunit of the voltage-dependent L-type gate calcium ion (Ca2+) channel Cav1.2, which regulates depolarization-dependent Ca2+ influx into the cell. It is unclear, however, by which mechanisms *CACNA1C* variants advance the risk of developing neuropsychiatric disorders (Gershon et al., 2014; Yoshimizu et al., 2015).

Several studies have confirmed *CACNA1C* to be a relevant genetic factor influencing human brain structure and function, such as gray matter volume (Kempton et al. 2009), functional coupling in the hippocampus (Erk et al., 2010), and neuronal processes involved in memory encoding and retrieval (Krug et al., 2014) in healthy subjects, but also affected individuals (Perrier et al., 2011). Moreover, *CACNA1C* has been implicated in behavioral changes relevant for neuropsychiatric disorders, such as emotion and mood, motivation, substance abuse and, in particular, cognitive functioning (for review see Kabir et al., 2016).

Cognitive deficits are a common feature of a wide spectrum of neuropsychiatric disorders, including, but not limited to, altered working memory and executive functioning in all phases of BD (Kurtz & Gerraty, 2009; Goldberg & Chengappa, 2009), low processing speed and negative affective bias in MDD (Hammar et al., 2003; Harmer et al., 2009), and a variety of cognitive deficits in SCZ (Barnett et al., 2010; for review see Millan et al., 2012). However, previous studies on *CACNA1C* in humans have yielded disparate results. For instance, carriers of the rs1006737 SNP risk allele have been reported to display poor verbal fluency (Krug et al., 2010), diminished working memory (Zhang et al. 2012), and impaired learning in general (Dietsche et al. 2014). Such alterations in cognitive functioning were linked to changes in functional

brain activation patterns, including altered dorsolateral prefrontal cortex activation during working memory tasks (Backes et al., 2014; Paulus et al., 2014). On the other hand, no significant impact on cognitive performance was found in healthy subjects by Roussos et al. (2011) and Soeiro-de-Souza et al. (2013). Likewise, Rolstad et al. (2016) did not find an association of *CACNA1C* risk alleles and cognitive performance in affected individuals. Finally, a different *CACNA1C* SNP, rs2007044, was found to negatively affect overall memory function (Cosgrove et al., 2017).

In mice, the results are similarly inconclusive. Because particularly high expression levels of the Cav1.2 channel are present in the hippocampus, Moosmang et al. (2005) assessed learning and memory in a mouse line with complete inactivation of the *Cacna1c* gene in hippocampus and neocortex and found impaired hippocampus-dependent spatial memory as assessed in the Morris water-maze. In contrast, White et al. (2008) did not obtain evidence for deficient memory acquisition in the Morris water-maze using mice lacking Cav1.2 channels in excitatory neurons of the hippocampus and cortex, but these mice displayed cognitive deficits in a probe trial one month after training, suggesting that Cav1.2 channels play an important role in remote spatial memories. During contextual fear learning, mice lacking Cav1.2 channels in hippocampus and cortex displayed intact fear conditioning and extinction (McKinney et al., 2008). In pairs of mice, however, observational but not classical fear learning was impaired when Cav1.2 channels were locally deleted in the anterior cingulate cortex of the observer, indicating that vicarious fear learning through social observation of a familiar conspecific requires Cav1.2 channels (Jeon et al., 2010). Yet other studies even suggested protective effects of *Cacna1c* deficiency. For instance, Zanos et al. (2015) reported *Cacna1c* haploinsufficiency to prevent object recognition deficits during aging. Together, these studies do not provide a consistent phenotype in mice, with background strain (Sittig et al., 2016), sex (Dao et al., 2010; Zanos et al., 2015), and age (Zanos et al., 2015) adding to the complexity of behavioral consequences on cognition elicited by *Cacna1c* deletions. These findings give reason to believe that within- as well as cross-species validation is necessary in order to obtain a more concise picture on *CACNA1C* genotype-phenotype relationships.

Research utilizing transgenic animal models has focused largely on mice in recent years (Homberg et al., 2017). However, the development of newer genetic approaches, for example, zinc-finger technology and CRISPR/Cas, opens the

examination of genes like *CACNA1C* up to other model organisms, such as rats. With their rich behavioral repertoire comprising enhanced social behavior, increased reward sensitivity, and more efficient learning strategies, rats represent an ideal complementary model system for cross-species validation of *Cacna1c* gene deletion effects (for review see Ellenbroek & Youn, 2016).

In this study, our aim was to advance understanding of the *Cacna1c* gene deletion effects on spatial learning and object memory in rats, and furthermore, to shed light on the influence *Cacna1c* has on reversal learning capabilities and cognitive flexibility which, to date, have not been examined in relevant animal models. We used a newly developed genetic *Cacna1c* rat model and compared wildtype littermate controls (*Cacna1c*$^{+/+}$) and constitutive heterozygous (*Cacna1c*$^{+/-}$) males and females. We hypothesized that reduced *Cacna1c* expression alters spatial memory abilities, reversal learning and object memory in *Cacna1c*$^{+/-}$ animals, as compared to their littermate controls.

MATERIALS AND METHODS

Animals and housing

Heterozygous *Cacna1c*$^{+/-}$ rats were generated using zinc finger technology by SAGE Labs (now Horizon Discovery Ltd, Cambridge, UK) on a Sprague-Dawley background, following a previously established protocol (Geurts et al., 2009). *Cacna1c*$^{+/-}$ rats carry a 4 base pair (bp) deletion at 460649-460652 bp in genomic sequence resulting in an early stop codon in exon 6. Previously, we have shown that Cav1.2 protein levels in the brain of *Cacna1c*$^{+/-}$ rats are reduced by ~50% (Kisko et al., submitted). Homozygous *Cacna1c*$^{-/-}$ rats were not used since they are embryonically lethal.

A heterozygous breeding protocol was used to obtain offspring from both genotypes. To this aim, Sprague-Dawley females obtained from Charles River (Sulzfeld, Germany) and male *Cacna1c*$^{+/-}$ rats were paired for breeding. Sprague-Dawley females were used because breeding efficacy is reduced in female *Cacna1c*$^{+/-}$ rats (not shown). In order to avoid litter effects, only litters with both genotypes and sexes were included in the experiments. Breeding was performed at the Faculty of Psychology, Philipps-University Marburg, Germany.

Approximately 2 weeks after pairing for breeding, females were individually housed and inspected daily for pregnancy and delivery. The day of birth was considered as

postnatal day (PND) 0. After weaning on PND 21, rats were socially housed in groups of 4-6 with same-sex partners in polycarbonate Macrolon Type IV cages (Tecniplast Deutschland GmbH, Hohenpeißenberg, Germany; 58 x 38 x 20 cm, length x width, x height) under standard laboratory conditions (22±2 °C and 40-70 % humidity) with free access to standard rodent chow and water. Rats were identified by paw tattoo, using non-toxic animal tattoo ink (Ketchum permanent tattoo inks green paste, Ketchum Manufacturing Inc., Brockville, Canada). The ink was inserted subcutaneously through a 30 gauge hypodermic needle tip into the center of the paw on PND 5±1.

After weaning, all animals were handled (PND 24±3) using a standard handling protocol. All procedures were conducted in strict accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and the relevant local or national rules and regulations of Germany and were subject to prior authorization by the local government (MR 20/35 Nr. 19/2014; Tierschutzbehörde, Regierungspräsdium Gießen, Germany).

Genotyping

Rat tail snips were collected by dissecting ~0.3 cm of tail on PND 5±1. Tails were digested, genomic DNA was isolated and purified using the Qiagen DNAeasy Blood & Tissue Kit according to the manufacturer's instructions (Hilden, Germany). After the extraction, 2.0 µl of DNA in buffer containing ~250-400 µg of DNA was amplified by PCR using the Promega PCR Master Mix (Mannheim, Germany). The following primers were used: GCTGCTGAGCCTTTTATTGG (*Cacna1c* Cel-1 F) and CCTCCTGGATAGCTGCTGAC (*Cacna1c* Cel-1 R). Genotyping was performed on a 3130xl Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA, USA).

Behavioral phenotyping

As part of our longitudinal and comprehensive deep behavioral phenotyping approach, object recognition, spatial memory, and reversal learning capabilities were assessed in male and female constitutive heterozygous *Cacna1c*$^{+/-}$ rats and compared to wildtype *Cacna1c*$^{+/+}$ littermate controls, with balanced representation of sexes in both groups. Novel object recognition was assessed in male and female *Cacna1c*$^{+/-}$ rats and *Cacna1c*$^{+/+}$ same-sex littermate controls on PND 92-94±1. Spatial learning and re-learning was performed on PND 117-131±13. Before entering these paradigms, all

animals were tested in other behavioral assays, namely playback of 50-kHz ultrasonic vocalizations, play behavior, repetitive behavior, olfactory habituation and dishabituation, open field, and elevated plus-maze (Kisko et al., submitted). Novel object recognition was tested in N=20 male and N=20 female *Cacna1c*$^{+/-}$ rats and N=20 male and N=20 female *Cacna1c*$^{+/+}$ littermates. Spatial and reversal learning was assessed in N=10 male and N=10 female *Cacna1c*$^{+/-}$ rats and N=10 male and N=10 female *Cacna1c*$^{+/+}$ littermates. Behavioral testing was performed by an experimenter blind to the animals' genotype and conducted during the light phase of a 12:12 light/dark schedule. Behavioral analysis was also performed by an experienced observer blind to experimental condition.

Novel object recognition

Around PND 92-94, the novel object recognition test was conducted in a large open field, as described previously (Bevins & Besheer, 2006; Valluy et al., 2015). The open field was made of gray plastic (60 x 60 x 60 cm) and rats were first habituated to the open field (no objects present) by placing them into the box for 20 min. Then, 24 h after the habituation session, the novel object recognition test was conducted, which consisted of three phases: acquisition trial, inter-trial interval, and recognition trial. In the acquisition trial, each rat was allowed to freely explore the open field containing two identical sample objects for 5 min. The objects were placed in one of the back corners of the box, with the objects situated 15 cm away from the walls. As objects, either two silver iron cylinders (5 cm in diameter, 8 cm high) or two red metal cubes (5 x 5 x 8 cm) were used in a counter-balanced manner. After the acquisition trial, the rats were returned to their home cages for 30 min, the inter-trial interval. During that time, one clean familiar object and one clean novel object were placed in the open field, where the two identical objects had been located during in the acquisition trial. After the inter-trial interval, each rat was returned to the open field for a 5 min recognition trial and allowed to freely explore the familiar and the novel object.

For behavioral analyses, a digital camera (EQ150, EverFocus, Taipei, Taiwan) was mounted 1.5 m above the floor of the open field and connected to a personal computer for recording and data storage. Object exploration was quantified as time spent sniffing the object and scored whenever the nose was oriented toward the object and the nose-object or front paw-object distance was 2 cm or less. Recognition memory was defined

as spending more time sniffing the novel object than the familiar object. Testing was performed under dim white light (16 lux) conditions.

Spatial and reversal learning

Around PND 120-130, rats were trained in the spatial learning and reversal learning tasks, using a modified protocol previously established (Görisch & Schwarting, 2006). Spatial and reversal learning was performed on a radial eight arm maze made of black plastic. The arms (9.8 x 40.5 cm) extended radially from a central platform (diameter: 24 cm) and were numbered in a clock-wise fashion from 1 to 8. Each arm had a single transparent plastic wall (20 x 17 cm) affixed to its right side to restrict rats from traversing to adjacent arms without entering the central platform. Four cm from the distal end of each arm, a food pit (5.3 cm in diameter; 4.0 cm deep) was embedded into its floor. The maze was positioned 52 cm above the floor in a testing room with several extra-maze cues. To eliminate distinct odor cues from the baited arms, four containers with food pellets were placed on the floor beneath the center of the maze. To enhance the incentive of food rewards, rats were food deprived, with food being withdrawn from home cages except for 1 h of daily free access. For food deprivation, rats were socially isolated in a Makrolon type III cage (265 x 150 x 425 mm, plus high stainless-steel covers and wood stick; Tecniplast Deutschland GmbH). Food deprivation and social isolation started seven days before the beginning of the spatial and reversal learning task. Starting with the day of radial maze training, the animals received their daily 1 h free access to food in their home cage no earlier than 1 h after spatial learning or reversal learning.

The spatial learning period lasted seven days, immediately followed by the reversal learning period, which also lasted seven days. Initially, the rats were exposed to the food pellets later used as reward (45 mg, BioServ Dustless Precision Pellets, Flemington, NJ, USA) in the home cage and were habituated to the radial eight arm maze, which then contained food pellets in all arms, both on the arms and in the food pits. During habituation, a given animal remained on the maze until it had eaten all pellets or until a cut-off criterion of 30 min was reached. During the seven spatial learning days, the rats were tested in five trials per day. For all animals, and during all trials of each spatial learning day, arms 1, 3, 5, 6, and 8 never contained food, whereas arm 2 was consistently baited with six food pellets and arms 4 and 7 were consistently

baited with one pellet. On day seven of the spatial learning period, a sixth and last trial was conducted as a probe trial with no food pellets available, lasting 5 min.

During the seven reversal learning days, the rats were again tested during five trials per day. Importantly, however, for all animals, and during all trials of each reversal learning day, arms 1, 2, 4, 6, and 7 never contained food, whereas arm 5 was consistently baited with six pellets and arms 3 and 8 were consistently baited with one food pellet. On day seven of the reversal learning period, a sixth trial was conducted as a probe trial with no available food pellets, lasting 5 min.

Between trials, the animal was placed singly into a home cage and started its next trial only after all other animals from that group had had their turn. The order animals were tested in was rotated randomly each day. A trial was ended only if the animal had found and eaten all food pellets or if a cut-off criterion of 5 min was reached (time to criterion). Start positions were rotated, with rats starting from new positions on the central platform in each trial. The maze was cleaned with 0.1% acetic acid and dried thoroughly before each trial.

Behavior was monitored via video camera (Panasonic, Ultrak CCTV Lens, Japan) from about 150 cm above the radial maze, which fed into an external multimedia hard drive (ScreenPlay Pro HD, Iomega). For behavioral analysis, an experienced observer scored the videos for the type of arm entries (counted if all four paws were placed on that arm) and the time until a trial was completed. Arm entries were scored as either a) correct entries (number of baited arms visited and emptied, max. 3) b) errors of reference memory (“RM”, initial entries into non-baited arms, as well as entries into baited arms without bait collection), c) errors of working memory (“WM”, repeated entries into baited arms) or d) “mixed” errors (“MIX”, repeated entries into non-baited arms) for both spatial learning and reversal learning periods. In addition, on reversal learning day 1, arm entries were scored for previously baited arms (i.e. arms baited during the spatial learning period), currently baited arms (i.e. arms baited during the reversal learning period), and never baited arms. Spatial learning and reversal learning was tested under dim white light (70 lux) conditions.

Statistical analysis

All statistical tests were carried out using IBM SPSS Statistics (Version 24.0) software. Tests were performed for males and females, separately. For the analysis of time to

criterion, average number of entries and errors, ANOVAs for repeated measurements were calculated with the within-subject factor day of learning (1 to 7) and between-subject factor genotype (*Cacna1c*$^{+/+}$ vs. *Cacna1c*$^{+/-}$). Error counts were always averaged for each day of learning and converted into percentages of made entries. For the comparison of day 1 and 7 of learning within each genotype, t-tests for paired samples were conducted. Arm preference during the probe trials was analysed using paired one-tailed t-tests, comparing entries into baited and entries into non-baited arms for each of the four experimental groups. Differences in preference between genotypes were assessed by independent sample t-tests. For the analysis of cognitive flexibility on reversal learning day 1, entries into previously, currently and never baited arms were compared to chance level by the means of one-sample one-tailed t-tests. One-tailed paired t-tests were used for the comparison of higher and lower rewarded arms, with the latter score being the average of both of the two lower rewarded arms. For the assessment of novel object recognition, percentages of time spent sniffing the familiar vs. the novel object were compared using paired one-tailed t-tests. Genotype differences in general exploration of all objects were analysed with independent sample t-tests. A p-value of < 0.050 was considered statistically significant. All values were reported as mean ± standard error means (SEM).

RESULTS

Spatial learning

Males: Spatial learning in males was reflected in reduced time to criterion over the seven-day period, irrespective of genotype (day: $F_{6,108}=12.469$; $p<0.001$; genotype: $F_{1,18}=0.654$; $p=0.429$; day x genotype: $F_{6,108}=0.182$; $p=0.981$). Both *Cacna1c*$^{+/+}$ and *Cacna1c*$^{+/-}$ males were faster on day 7 compared to day 1 ($t_9=3.885$; $p=0.004$ and $t_9=3.649$; $p=0.005$; respectively) [Figure 1A, left]. Spatial learning was also reflected in a reduction in arm entries per trial (day: $F_{6,108}=6.967$; $p<0.001$; genotype: $F_{1,18}=1.743$; $p=0.203$; day x genotype: $F_{6,108}=0.886$; $p=0.508$), with only *Cacna1c*$^{+/+}$ males displaying a reduction from day 1 to day 7 ($t_9=2.544$; $p=0.032$) but not *Cacna1c*$^{+/-}$ littermates ($t_9=1.386$; $p=0.199$) [Figure 1C, left].

In males, both genotypes displayed spatial learning capabilities, as reflected in reduced wrong arm entries as days of training increased (day: $F_{6,108}=9.137$; $p<0.001$; genotype: $F_{1,18}=0.034$; $p=0.855$; day x genotype: $F_{6,108}=0.791$; $p=0.579$), with both

genotypes making fewer wrong entries on day 7 compared to day 1 (+/+: t_9=3.515 p=0.007; +/-: t_9=4.996; p=0.001) [Figure 2A, left]. To differentiate between different memory components, memory errors were then analyzed separately for reference memory errors (RM) reflecting errors in the actual long-term learning of the arm configuration, working memory errors (WM), as a measure of short-term memory of which arms had already been entered in a specific trial, and mixed memory errors (MIX), as a combination of the two aforementioned errors, which were counted whenever an animal entered an arm that was not rewarded and had furthermore already been entered in that trial.

Rats made fewer RM errors over training days (day: $F_{6,108}$=3.851; p=0.002; genotype: $F_{1,18}$=0.749; p=0.398; day x genotype: $F_{6,108}$=0.466; p=0.832). The reduction from day 1 to 7 was most prominent in *Cacna1c*$^{+/-}$ (t_9=2.366; p=0.042) and less pronounced in *Cacna1c*$^{+/+}$ males (t_9=1.684; p=0.126) [Figure 2C left]. On WM errors, i.e. repeated entries into rewarded arms, training day had no effect on either group (day: $F_{6,108}$=1.493; p=0.187; genotype: $F_{1,18}$=1.846; p=0.191; day x genotype: $F_{6,108}$=0.398; p=0.879) even when comparing the first and last day of testing (+/+: t_9=-0.330; p=0.749; +/-: t_9=-0.079; p=0.939) [Figure 3A, left]. Repeated entries into unrewarded arms, i.e. MIX errors, were made more rarely as training days increased (day: $F_{6,108}$=10.978; p<0.001; genotype: $F_{1,18}$=2.836; p=0.109; day x genotype: $F_{6,108}$=1.227; p=0.298). Both *Cacna1c*$^{+/+}$ and *Cacna1c*$^{+/-}$ males made fewer mistakes on day 7 than day 1 (t_9=6.013; p<0.001 and t_9=4.554; p=0.001; respectively) [Figure 3C, left].

During the following probe trial, only *Cacna1c*$^{+/-}$ (t_8=3.701; p=0.003) but not *Cacna1c*$^{+/+}$ males (t_9=1.354; p=0.105) preferred the previously rewarded arms to the non-rewarded arms. Genotypes differed in the proportion of entries made into the rewarded arms, with a higher score for *Cacna1c*$^{+/-}$ males (rewarded: t_{17}=1.751; p=0.049; non-rewarded: t_{17}= -1.627; p=0.061) [Figure 4A].

Females: In females, both genotypes were able to acquire the rewarded arm configuration over the course of seven days. An effect of training day was found for time to criterion (day: $F_{6,108}$=37.014; p<0.001; genotype: $F_{1,18}$=0.403; p=0.533; day x genotype: $F_{6,108}$=1.118; p=0.357), with both *Cacna1c*$^{+/+}$ and *Cacna1c*$^{+/-}$ females needing less time on the last day of spatial learning compared to the first (t_9=10.558; p<0.001 and t_9=5.811; p<0.001; respectively) [Figure 1B, left]. An effect of both training

day and genotype, as well as an interaction was found for the average number of entries made during a trial (day: $F_{6,108}$=7.027; p<0.001; genotype: $F_{1,18}$=13.123; p=0.002; day x genotype: $F_{6,108}$=2.259; p=0.043). *Cacna1c*$^{+/-}$ females were, in general, less active than their *Cacna1c*$^{+/+}$ littermates and showed signs of hypo-activity during the first few days of testing. The reduction of made entries from day 1 to day 7 was only significant for *Cacna1c*$^{+/+}$ females (+/+: t_9=6.070; p<0.001; +/-: t_9=0.963; p=0.361) [Figure 1D, left].

In females, both genotypes reduced the number of wrong arm entries across days of training (day: $F_{6,108}$=25.383; p<0.001; genotype: $F_{1,18}$=1.706; p=0.208; day x genotype: $F_{6,108}$=1.576; p=0.161). *Cacna1c*$^{+/+}$ as well as *Cacna1c*$^{+/-}$ females made fewer errors on day 7 than at the beginning (t_9=5.971; p<0.001 and t_9=4.399; p=0.002; respectively) [Figure 2B, left]. The same was true for RM errors, i.e. initial entries into unrewarded arms (day: $F_{6,108}$=15.028; p<0.001; genotype: $F_{1,18}$=0.145; p=0.708; day x genotype: $F_{6,108}$=1.733; p=0.120) where both genotypes improved from day 1 to the last spatial learning day (+/+: t_9=5.040; p=0.001; +/-: t_9=3.143; p=0.012) [Figure 2D, left]. Regarding WM errors, no progress was seen for either genotype between day 1 and day 7 (+/+: t_9=0.699; p=0.502; +/-: t_9=-0.712; p=0.495), although an effect of training day was found to be significant (day: $F_{6,108}$=3.206; p=0.006; genotype: $F_{1,18}$=0.052; p=0.823; day x genotype: $F_{6,108}$=0.462; p=0.835) [Figure 3B, left]. However, both experimental groups improved their MIX error performance, in that they made less re-entries into non-rewarded arms across training days (day: $F_{6,108}$=10.337; p<0.001; genotype: $F_{1,18}$=8.836; p=0.008; day x genotype: $F_{6,108}$=0.268; p=0.951) and on day 7 compared to day 1 (+/+: t_9=3.459; p=0.007; +/-:t_9=3.428; p=0.008). Here, *Cacna1c*$^{+/-}$ females performed better than their *Cacna1c*$^{+/+}$ counterparts ($F_{1,18}$=8.836; p=0.008) [Figure 3D, left].

In the probe trial, both *Cacna1c*$^{+/+}$ and *Cacna1c*$^{+/-}$ females successfully distinguished between previously rewarded and non-rewarded arms (t_9=4.279; p=0.001 and t_9=3.277; p=0.005; respectively), with no genotype difference in the preference of either arm type (rewarded: t_{18}=0.729.; p=0.238; non-rewarded: t_{18}=-0.676; p=0.254) [Figure 4B].

Reversal learning

Males: During the seven-day reversal learning phase, similar activity results were obtained for both *Cacna1c*$^{+/+}$ and *Cacna1c*$^{+/-}$ males. Irrespective of genotype, an effect for training day on time to criterion was found (day: $F_{6,108}$=11.214; p<0.001; genotype: $F_{1,18}$=1.701; p=0.209; day x genotype: $F_{6,108}$=0.652; p=0.688). Both genotypes took less time to complete the task on day 7 than six days previously (+/+: t_9=3.884; p=0.004; +/-: t_9=4.140; p=0.003) [Figure 1A, right]. The number of average entries did not reduce in the same way between the first and last day (+/+: t_9=1.347; p=0.211; +/-: t_9=1.138; p=0.284;), even though a main effect of training day was found (day: $F_{6,108}$=4.543; p<0.001; genotype: $F_{1,18}$=0.654; p=0.429; day x genotype: $F_{6,108}$=0.450; p=0.843) [Figure 1C, right].

Cacna1c$^{+/+}$ and *Cacna1c*$^{+/-}$ males learned to enter the correct, newly baited arms over the seven-day reversal learning period, displayed by the reduced percentage of wrong entries (day: $F_{6,108}$=12.479; p<0.001; genotype: $F_{1,18}$=0.574; p=0.459; day x genotype: $F_{6,108}$=0.674; p=0.671), a change that was also apparent between days 1 and 7 of this second learning phase (+/+: t_9=3.760; p=0.004; +/-: t_9=4.214; p=0.002) [Figure 2A, right]. Split by error type, the same pattern was found for RM errors (day: $F_{6,108}$=11.876; p<0.001; genotype: $F_{1,18}$=1.147; p=0.298; day x genotype: $F_{6,108}$=0.252; p=0.958) where both genotypes made fewer errors by day 7 (+/+: t_9=5.071; p=0.001; +/-: t_9=3.310; p=0.009) [Figure 2C, right]. Again, no effect of training day or genotype was found regarding WM errors (day: $F_{6,108}$=0.536; p=0.780; genotype: $F_{1,18}$=0.669; p=0.424; day x genotype: $F_{6,108}$=0.445; p=0.847). Neither group made fewer WM errors after reversal learning than at the very start (+/+: t_9=-1.100; p=0.300; +/-: t_9=-0.693; p=0.506) [Figure 3A, right]. In contrast, there was an effect of learning day on MIX errors (day: $F_{6,108}$=5.157; p<0.001; genotype: $F_{1,18}$=0.125; p=0.728; day x genotype: $F_{6,108}$=1.522; p=0.178). *Cacna1c*$^{+/+}$ as well as *Cacna1c* $^{+/-}$ males reduced the number of MIX errors, relative to all entries in the specific trial between day 1 and 7 of reversal learning (t_9=2.343; p=0.044 and t_9=2.138; p=0.061; respectively) [Figure 3C, right].

In the probe trial following reversal learning, both genotypes could distinguish between the previously rewarded arms and the non-rewarded arms (+/+: t_8=1.973; p=0.042; +/-: t_9=3.701; p=0.003), with *Cacna1c*$^{+/-}$ males showing a stronger preference for the arms that were baited than their *Cacna1c*$^{+/+}$ littermates (t_{17}=2.265.; p=0.019) and less interest in the non-baited choices (t_{17}=-2.231; p=0.020) [Figure 4C].

Females: In females, both genotypes displayed intact reversal learning with a lower time to criterion on day 7 of reversal learning than on day 1 (+/+: t_9=2.874; p=0.018; +/-: t_9=3.968; p=0.003), as reflected in a main effect of learning day (day: $F_{6,108}$=17.068; p<0.001; genotype: $F_{1,18}$=0.144; p=0.708; day x genotype: $F_{6,108}$=0.915; p=0.487) [Figure 1B, right]. The same was true for average number of entries per trial (day: $F_{6,108}$=21.258; p<0.001; genotype: $F_{1,18}$=0.295; p=0.594; day x genotype: $F_{6,108}$=0.301; p=0.935). Both *Cacna1c*$^{+/+}$ and *Cacna1c*$^{+/-}$ were less active on the last learning day than on the first (+/+: t_9=3.411; p=0.008; +/-: t_9=5.219; p=0.001) [Figure 1D, right].

Reversal learning capabilities also became apparent in the reduction of erroneous entries over time (day: $F_{6,108}$=20.000; p<0.001; genotype: $F_{1,18}$=0.323; p=0.577; day x genotype: $F_{6,108}$=0.427; p=0.860), from day 1 to day 7 (+/+: t_9=3.626; p=0.006; +/-: t_9=4.787; p=0.001) [Figure 2B, right], which was also true for reference memory errors (+/+: t_9=4.087; p=0.003; +/-: t_9=3.704; p=0.005) including the main effect of learning day (day: $F_{6,108}$=16.720; p<0.001; genotype: $F_{1,18}$=0.173; p=0.683; day x genotype: $F_{6,108}$=0.145; p=0.990) [Figure 2D, right]. Concerning working memory, repeated entries into rewarded arms (WM errors) did not decrease over training days (day: $F_{6,108}$=1.452; p=0.202; genotype: $F_{1,18}$=0.725; p=0.406; day x genotype: $F_{6,108}$=0.572; p=0.752) and neither *Cacna1c*$^{+/+}$ nor *Cacna1c*$^{+/-}$ females improved from the first to the last day of training (t_9=1.391; p=0.198 and t_9=1.037; p=0.327; respectively) [Figure 3B, right]. For repeated entries into non-baited arms an effect of training day was found irrespective of genotype (day: $F_{6,108}$=9.993; p<0.001; genotype: $F_{1,18}$=0.195; p=0.664; day x genotype: $F_{6,108}$=0.554; p=0.766), although a look at day 1 vs. day 7 revealed that only the *Cacna1c*$^{+/-}$ females made fewer MIX errors on day 7 than on day 1 (t_9=3.838; p=0.004) whereas there was no change in the *Cacna1c*$^{+/+}$ females (t_9=1.774; p=0.110) [Figure 3D, right].

However, in the consecutive probe trial both genotypes displayed the ability to distinguish previously baited from non-baited arms (+/+: t_8=3.000; p=0.009; +/-: t_9=6.029; p<0.001), with a slightly more pronounced avoidance of the incorrect arms in *Cacna1c*$^{+/-}$ rats (baited: t_{17}=1.735; p=0.051 and non-baited: t_{17}=-2.076; p=0.027) [Figure 4D].

Cognitive flexibility

Males: Cognitive flexibility was assessed on reversal learning day 1 by comparing the percentage of entries into currently, previously, and never baited arms to chance level (12.5% for 1 out of 8 arms). In males, only *Cacna1c*$^{+/+}$ rats preferred the newly baited arms above chance level (t_9=4.943; p<0.001) and showed reduced entries into the previously baited arms to chance level (t_9=0.300; p=0.386), whereas never baited arms were entered at a probability below chance in both *Cacna1c*$^{+/+}$ (t_9=-3.100; p=0.007) and *Cacna1c*$^{+/-}$ males (t_9=-1.853; p=0.049). *Cacna1c*$^{+/-}$ males, however, entered both currently (t_9=-0.057; p=0.478) and previously rewarded arms (t_9=1.658; p=0.066) at chance level and did not show a preference for the new arm configuration on the first day [Figure 5A].

Females: Currently baited arms were preferred above chance level by both *Cacna1c*$^{+/+}$ (t_9=2.308; p=0.023) and *Cacna1c*$^{+/-}$ (t_9=5.088; p<0.001) females. Likewise, never baited arms were entered below chance probability by both genotypes (+/+:t_9=-6.527; p<0.001; +/-: t_9=-4.625; p<0.001). Regarding previously baited arms, *Cacna1c*$^{+/-}$ females already showed a disinterest equivalent to chance level (t_9=1.728; p=0.059) on the first day of reversal learning, while their *Cacna1c*$^{+/+}$ counterparts still preferred them to chance (t_9=2.963; p=0.008) [Figure 5B].

Reward sensitivity

Males: To test whether genotypes differed in reward sensitivity known to affect spatial learning in the radial arm maze (Görisch & Schwarting, 2006), entries made into the differently rewarded arms were compared. In males, a clear preference for the higher baited arms was evident in both genotypes during the spatial learning probe trial (+/+: t_9=4.583; p<0.001; +/-: t_9=2.414; p=0.020) [Figure 6A], but only in *Cacna1c*$^{+/-}$ (t_9=6.194; p<0.001) and not *Cacna1c*$^{+/+}$ rats (t_8=1.155; p=0.141) in the reversal learning probe trial [Figure 6C].

Females: The same holds true for females, where *Cacna1c*$^{+/+}$ and *Cacna1c*$^{+/-}$ rats preferred the higher baited arms during spatial earning probe (t_9=3.059; p=0.007 and t_9=6.228; p<0.001; respectively) [Figure 6B]. In the reversal learning probe, *Cacna1c*$^{+/+}$ females no longer entered the higher baited arm more frequently than the lower baited

arms, whereas their *Cacna1c*$^{+/-}$ siblings still did (+/+: t_8=2.121; p=0.034; +/-: t_9=4.605; p<0.001) [Figure 6D].

Novel object recognition

In males and in females, neither *Cacna1c*$^{+/+}$ nor their *Cacna1c*$^{+/-}$ littermates had difficulties distinguishing the novel from the familiar object in the second trial (males: t_{19}=3.192; p=0.003 and t_{19}=2.131; p=0.023; females: t_{19}=1.986; p=0.031 and t_{18}=2.106; p=0.025; respectively) [Figure 7A]. In both sexes, there was also no difference in general exploration time for either trial 1 (males: t_{38}=1.491; p=0.144; females: t_{38}=0.104; p=0.918) [Figure 7B, left] or trial 2 (males: t_{38}=0.731; p=0.470; females: t_{37}=1.582; p=0.122) [Figure 7B, right].

DISCUSSION

Because *Cacna1c* is strongly implicated in multiple neuropsychiatric disorders characterized by deficits in cognitive functioning, our goal was to use a newly developed heterozygous *Cacna1c* rat model to examine, among others, the gene's role in cognition. Specifically, this study set out to investigate spatial and reversal learning, as well as object recognition memory in heterozygous *Cacna1c*$^{+/-}$ rats in comparison to wildtype *Cacna1c*$^{+/+}$ littermate controls in both sexes. Our results show that both *Cacna1c*$^{+/+}$ and *Cacna1c*$^{+/-}$ animals were able to learn the rewarded arm configuration of a radial maze over the course of 7 days. Both groups also showed reversal learning patterns indicative of intact abilities. In females, genotype differences were evident in the initial spatial learning phase, with *Cacna1c*$^{+/-}$ females showing hypo-activity and fewer mixed errors. In males, a difference was found during probe trials for both learning phases, with *Cacna1c*$^{+/-}$ rats displaying better distinction between previously baited and non-baited arms; and regarding cognitive flexibility in favor of the *Cacna1c*$^{+/+}$ animals. All experimental groups proved to be sensitive to reward magnitude and fully able to distinguish between novel and familiar objects in the novel object recognition task.

Spatial Learning

In males, both *Cacna1c*$^{+/+}$ and *Cacna1c*$^{+/-}$ rats showed intact spatial learning abilities. The time to criterion decreased in both genotypes with days of training, showing that

the animals entered the correct arms sooner as time progressed. Regarding the number of arm entries, a significant reduction was observed for *Cacna1c*$^{+/+}$ males, whereas *Cacna1c*$^{+/-}$ males did not enter fewer arms on spatial learning day 7 than on day 1. However, and most importantly, the percentage of wrong arm entries reduced in both groups, indicating increasing accuracy and unimpaired overall spatial learning capabilities for both genotypes.

When comparing our results with the existing literature on *Cacna1c*, one needs to distinguish between spatial reference memory and spatial working memory, as the radial arm maze task used in the present study is known to assess both, whereas paradigms, such as the spontaneous alteration in T- and Y-mazes, usually concentrate on spatial working memory, while other tests, like the Morris water-maze, primarily examine reference memory (Morellini, 2013).

We took a detailed look at individual error types and descriptively found a decrease in reference memory errors across training days for both genotypes, but the reduction from first to last day of spatial learning was only significant in *Cacna1c*$^{+/-}$ males. This effect was most likely caused by the lower initial error percentage in *Cacna1c*$^{+/+}$ males, which may have allowed less potential for further improvement. However, during the probe trial after seven days of learning, only *Cacna1c*$^{+/-}$ males were able to successfully distinguish previously rewarded and unrewarded arms due to a higher preference for the previously baited arms, implying that they had developed a robust memory trace of the reward locations.

This latter finding stands in contrast to a study by Moosmang et al. (2005), who reported no difference in initial improvement of both wildtype controls and conditional knockout mice with inactivation of *Cacna1c* in the hippocampus and neocortex in the Morris water-maze task. While controls improved further, the conditional knockout mice showed a severe learning impairment after the second day and significantly less effective search strategies in another maze. Moreover, the differences between both genotypes were no longer observed in the two probe trials following one and two weeks after training, which raises the question of whether the dissimilarity witnessed in our *Cacna1c*$^{+/+}$ and *Cacna1c*$^{+/-}$ males is something mitigated by passing time, or if in our case, the mechanism is different in nature. The latter notion is supported by findings made by White et al. (2008), who examined a genetic mouse model similar to the one used by Moosmang et al. (2005) in the Morris water-maze, but administered probe

trials during testing, like we did, as well as a remote memory probe after 30 days. They, like us, found no major differences in learning performance between both genotypes. In direct opposition to the hypothesis that a discrepancy in probe trial findings was merely due to timing differences, the authors found no difference between genotypes in the probe trials directly following the training sessions, but significantly worse performance in *Cacna1c*$^{+/-}$ mice during the second, more remote probe, indicating more robust memory traces in wildtype animals after several weeks had passed. However, a study performed by Temme et al. (2016) paints a slightly different picture. Their results are partially in accordance with what we found in our rat model, in that both wildtype and conditional knockout mice with neuron-specific deletion of Cav1.2 performed well in the Morris water-maze. Interestingly, the authors further demonstrated that the difficulty of the task appears to have an effect on the emergence of genotype differences. Specifically, when increasing task difficulty by providing only limited cues for navigation, significant deficits were found in the neuron-specific knockout animals. Dedic et al. (2017) used the water-cross maze, a paradigm similar to the Morris water-maze, yet advantageous in its simplicity, in an examination of male heterozygous and forebrain-specific *Cacna1c* knockout mice. Their observations directly oppose those made by us in that the knockout mice in their study showed a radical impairment of spatial learning abilities, but enhanced cognitive flexibility and were equal to wildtype animals in a subsequent probe trial. In a further spatial object recognition test, no genotype differences were observed. Since this test requires object, as well as spatial memory, no direct comparisons can be drawn. Kabitzike et al. (2017) had male wildtype and heterozygous mice undergo a procedural T-maze paradigm and found no genotype differences in performance. These results support our findings, and this is the only other spatial learning study we know of that used constitutive heterozygous animals. It has to be said, though, that Kabitzike et al. (2017) employed a paradigm that requires egocentric orientation, as opposed to allocentric spatial navigation used in all other previously mentioned studies.

In terms of pure working memory errors, a significant reduction could not be found for *Cacna1c*$^{+/+}$ nor *Cacna1c*$^{+/-}$ animals, although this is likely due to the fact that this type of error was committed most infrequently to begin with. The notion that the heterozygous *Cacna1c* genotype does not influence working memory is in accordance with findings by Zanos et al. (2015) who tested male and female *Cacna1c*$^{+/+}$ and

Cacna1c$^{+/-}$ mice in a Y-maze paradigm and did not discover an effect of genotype on performance. Bavley et al. (2017), who employed a non-reward based spontaneous T-maze alternation task in male *Cacna1c*$^{+/+}$ and *Cacna1c*$^{+/-}$ mice, likewise did not find genotype differences in working memory performance. Only when exposing mice to chronic unpredictable stress, genotype differences emerged in favor of the heterozygous *Cacna1c*$^{+/-}$ mice, which appeared to be more resilient.

In females, the behavioral pattern was quite similar to that of males. *Cacna1c*$^{+/-}$ females and their *Cacna1c*$^{+/+}$ littermates both showed a reduction in time needed to complete the task, as well as arms that were entered until either the success or time criterion were met, although the average number of arms was lower for *Cacna1c*$^{+/-}$ animals initially, suggesting slight hypo-activity in the very beginning of spatial learning, which also explains the difference in arm entry reduction observed in comparison to *Cacna1c*$^{+/+}$ rats. Nevertheless, *Cacna1c*$^{+/-}$ were on par with *Cacna1c*$^{+/+}$ females in terms of accuracy. Both genotypes made fewer errors with time, and this applied to reference memory, as well as mixed memory errors, where *Cacna1c*$^{+/-}$ females performed better, but not to working memory errors. Once again, the latter findings can most likely be explained by floor effects. Both female groups were able to identify the previously rewarded arms and the arms containing no reward in the probe trial after seven consecutive learning days. Regarding working memory performance, the results obtained in females mirror those in males, in that they did not show any improvement across training days as well as no differences between genotypes. These results are in accordance with the findings by Zanos et al. (2015). Unfortunately, most other existing studies on spatial learning examined males only, and those that included females pooled data from both sexes (White et al., 2008; Temme et al., 2016).

Reversal Learning

During the reversal learning phase that directly followed the seven days of spatial learning, both genotypes showed intact reversal learning abilities in males, with initial perseveration trends in *Cacna1c*$^{+/-}$ rats. *Cacna1c*$^{+/-}$ males, as well as their *Cacna1c*$^{+/+}$ littermates, displayed the expected pattern of increases in time to criterion, number of arm entries and total errors on reversal learning day 1, which then decreased significantly again over the course of this second seven-day phase. There were no genotype differences in the improvement of total number of errors, reference memory

or mixed memory errors, suggesting that reversal learning capabilities in general are intact in heterozygous *Cacna1c*$^{+/-}$ males.

An additional readout of cognitive flexibility was assessed on reversal learning day 1 by comparing the entries made into the newly and currently baited arms, i.e. those that had been baited previously during spatial learning and the two arms that had never been baited. In males, only wildtype *Cacna1c*$^{+/+}$ animals managed the transition to the new arm configuration seamlessly, while their *Cacna1c*$^{+/-}$ littermates did not enter the currently baited arms at a frequency above chance level, while descriptively still preferring the previously baited arms. This finding points to perseveration tendencies in *Cacna1c*$^{+/-}$ males, which appear to be short-term, however, as proven by their reversal learning performance and superiority during the following five minute probe trial. In the reversal learning probe, *Cacna1c*$^{+/-}$ males again preferred the correct arms and avoided the non-rewarded ones to a greater extent than their siblings, although both groups were able to distinguish between both arm types successfully. This suggests that male *Cacna1c*$^{+/-}$ rats, even though showing a slight retardation on the first day, adopt the new reward configuration as a robust memory trace after seven days of reversal learning, and again learn it more thoroughly than their *Cacna1c*$^{+/+}$ littermates.

In females, a similar pattern indicating intact reversal learning was found. There was no genotype difference in the reduction of time spent on the maze and arm entries to completion, which both genotypes displayed. Likewise, both genotypes made fewer errors in general over time, and fewer reference and mixed memory error, again with the aforementioned exception of working memory errors, which were low to begin with and were not reduced until the end of reversal learning. In contrast to males, in females both genotypes were able to distinguish between rewarded and non-rewarded arms in the reversal learning probe, although in females, too, there was a genotype difference in the avoidance of the non-baited arms, with heterozygous animals entering those arms less often in proportion to all entries.

Heterozygous *Cacna1c*$^{+/-}$ females showed the ideal pattern of cognitive flexibility on reversal learning day 1, entering the newly baited arms above chance, the never baited below chance and the previously baited at chance level, whereas their wildtype *Cacna1c*$^{+/+}$ siblings still enter the previously baited, no longer relevant arms above chance level. Seeing that in males, this relationship is almost vice versa, this finding

again points towards differential mechanisms in how *Cacna1c* affects cognition and behavior in the two sexes. In the course of the following reversal learning days, the wildtype females, however, adapted to the new configuration and did not commit more erroneous entries than their heterozygous littermates, indicating that rather than an impairment, reduced *Cacna1c* expression might simply delay reversal learning in females.

Two previous mouse studies have examined the effects of *Cacna1c* on cognitive flexibility and reversal learning and have yielded disparate results. Dedic et al. (2017) included a relearning trial in their water-cross maze paradigm performed on conditional forebrain knockout mice, and found an initial superiority of the male knockouts compared to wildtype controls regarding cognitive flexibility, which vanished after 30 days. This finding stands in direct contrast to the mild perseveration tendencies observed in our male *Cacna1c*$^{+/-}$ rats. On the other hand, Kabitzike et al. (2017) found a trend towards an impairment of flexibility in male heterozygous mice in the procedural T-maze test. Taken together, this supports the notion that *Cacna1c* expression levels might not have a linear and maybe even sex-specific relationship with impairments in spatial learning and relearning.

Reward Sensitivity

As a control for motivation, we tested whether reward sensitivity was impaired in *Cacna1c*$^{+/-}$ animals and found that experimental groups, regardless of sex or *Cacna1c* haploinsufficiency, could distinguish between higher and lower rewarded arms. It thus appears that differences in the spatial learning probe trial or reversal learning may not be explained by altered motivation. If anything, the *Cacna1c*$^{+/-}$ animals' reward sensitivity was slightly superior, seeing that they showed consistent preference for the higher rewarded arms in the probe trials of both phases. This finding argues against *Cacna1c* haploinsufficiency causing a depression-like phenotype, which is, among other symptoms, characterized by anhedonia and diminished motivation, as implied by human research (Lancaster et al., 2014). In mice, *Cacan1c* deletion was previously associated with an antidepressant phenotype (Dao et al., 2010; Kabir et al., 2016).

Object Recognition

In the present study, we also investigated novel object recognition, a memory task without spatial component. All experimental groups showed similar general exploration patterns of both objects during acquisition. Regarding actual discrimination performance during testing, *Cacna1c*$^{+/-}$ animals did not differ from their *Cacna1c*$^{+/+}$ littermates, either. Irrespective of sex, both genotypes were able to recognize the novel object as new in the second trial, demonstrated by more time spent sniffing the novel object compared to the familiar one. Irrespective of sex and genotype, rats preferred the novel object to the old one. At first glance, our finding stands in direct contrast to that of Zanos et al. (2015) who observed a significant correlation between *Cacna1c* expression in constitutive heterozygous mice and performance in the novel object recognition task, although, likewise, no difference between sexes was found, following an almost identical protocol. However, they investigated aging effects and the genotype difference found in their study was driven by an older group of mice that was about 17-18 months old. When comparing the age of mice (Dutta & Sengupta, 2016) and rats (Andreollo et al., 2012), it becomes clear that the rats we used were still comparatively young at 3 months and thus more equivalent in age to the group of young mice used by Zanos et al. (2015). These animals displayed intact novel object recognition, regardless of genotype. Further evidence that this reasoning is correct can be found in a study by Jeon et al. (2010) who, like us, did not find genotype differences in a mouse model with a conditional knockout of *Cacna1c* in the anterior cingulate cortex and, likewise, used comparatively young animals. It appears, therefore, that *Cacna1c* haploinsufficiency might have a protective influence on object memory later in life when cognitive abilities are known to deteriorate, as suggested by Zanos et al. (2015). This approach should be investigated further by longitudinal studies that assess object memory over the course of life in the same animals. If this holds true, another aspect that will need to be clarified is whether the age factor is dependent on molecular changes occurring with age or a different response in *Cacna1c*$^{+/-}$ animals to experiences related to object memory, such as repeated exposure to different stimuli.

Limitations and future perspectives

In general, most findings of previous studies support the notion that knockout mice - if at all different - perform worse in matters concerning spatial learning, and several findings diverge from those obtained in our study. This can have many reasons.

Obviously, species differences could play a role, as in contrast to our rat study, most of the existing literature focuses on mice. However, one distinctive feature of these studies is their use of region- or cell-specific knockouts. In most experiments, *Cacna1c* was deleted in task-relevant regions, such as the forebrain/PFC, hippocampus, or in neurons, exclusively, and only very few authors employed similar constitutive haploinsufficiency models, such as Kabitzike et al. (2017) or Zanos et al. (2015), suggesting that expression patterns play a key role. Furthermore, the diverging results give rise to the question of whether the effects *Cacna1c* has on cognition might vary with expression levels. It appears likely that a complete inactivation of the *Cacna1c* gene in hippocampus and neocortex leads to memory impairments, such as seen in the study by Moosmang et al. (2005), while full body haploinsufficiency has no deteriorating effects.

Other reasons for conflicting results may include the setup and requirements of the employed paradigms, none of which are directly comparable to the radial maze task we used, or the difference in the nature of the reward to be sought out by the animals. Most of the previous studies used an escape to perceived safety (e.g. in the Morris water-maze or the water-cross maze) as a goal for the animals to achieve, whereas the ingestion of the food pellets was the prime motivator in our experiment. If animals of different ages were investigated, as done in Zanos et al. (2015), a more elaborate result pattern might emerge. Also, the inclusion of remote probe trials is highly recommended for future studies. Furthermore, results on *Cacna1c* females, in general, are still sparse. Another point is the investigation of additional influences on performance outcomes, such as stress and aversive early life experiences, which have been shown to increase the chances of developing depressive symptoms later in life (Widom et al., 2007), sometimes interacting substantially with genetic risk factors (Caspi et al., 2003), as well as beneficial environmental influences like social support known to promote resilience to affective disorders (Kendler et al., 2005; Kaufman et al., 2006).

CONCLUSION

In summary, our results show for the first time intact spatial memory and reversal learning capabilities in a constitutive *Cacna1c* heterozygous rat model with impairments of initial cognitive flexibility but better long-term learning in *Cacna1c*$^{+/-}$

males and initial hypo-activity at a slightly better performance in *Cacna1c*$^{+/-}$ females. Reward sensitivity and object recognition abilities were not impaired in either sex, regardless of *Cacna1c* expression levels. Taken together, these results indicate that *Cacna1c* haploinsufficiency has a minor, but positive impact on (spatial) memory functions in rats.

DECLARATIONS:

List of Abbreviations:

bipolar disorder (BPD); major depressive disorder (MDD); schizophrenia (SCZ); single-nucleotide polymorphism (SNP); genome-wide association study (GWAS); heterozygous *Cacna1c* (*Cacna1c*$^{+/-}$); wildtype *Cacna1c* (*Cacna1c*$^{+/+}$); postnatal day (PND).

Ethics Approval: All procedures were conducted in strict accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and the relevant local or national rules and regulations of Germany and were subject to prior authorization by the local government (MR 20/35 Nr. 19/2014; Tierschutzbehörde, Regierungspräsdium Gießen, Germany).

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Authors contributions: MW conceived the study; MB, TK and DDV performed the experiments; MB analyzed the data; MB and MW wrote the manuscript; RS and MW acquired funding. All authors read and approved the final manuscript.

REFERENCES

Andreollo, N. A., Santos, E. F. dos, Araújo, M. R., Lopes, L. R., & others. (2012). Rat's age versus human's age: What is the relationship? *ABCD. Arquivos Brasileiros de Cirurgia Digestiva (São Paulo)*. Retrieved from http://repositorio.unicamp.br/handle/REPOSIP/25018

Backes, H., Dietsche, B., Nagels, A., Konrad, C., Witt, S. H., Rietschel, M., … Krug, A. (2014). Genetic variation in CACNA1C affects neural processing in major depression. *Journal of Psychiatric Research*, *53*, 38–46. https://doi.org/10.1016/j.jpsychires.2014.02.003

Barnett, J. H., Robbins, T. W., Leeson, V. C., Sahakian, B. J., Joyce, E. M., & Blackwell, A. D. (2010). Assessing cognitive function in clinical trials of schizophrenia. *Neuroscience & Biobehavioral Reviews*, *34*(8), 1161–1177. https://doi.org/10.1016/j.neubiorev.2010.01.012

Bavley, C. C., Fischer, D. K., Rizzo, B. K., & Rajadhyaksha, A. M. (2017). Ca v 1.2 channels mediate persistent chronic stress-induced behavioral deficits that are associated with prefrontal cortex activation of the p25/Cdk5-glucocorticoid receptor pathway. *Neurobiology of Stress*, *7*, 27–37. https://doi.org/10.1016/j.ynstr.2017.02.004

Bevins, R. A., & Besheer, J. (2006). Object recognition in rats and mice: a one-trial non-matching-to-sample learning task to study "recognition memory". *Nature Protocols*, *1*(3), 1306–1311. https://doi.org/10.1038/nprot.2006.205

Caspi, A., Sugden, K., Moffitt, T. E., Taylor, A., Craig, I. W., Harrington, H., … Poulton, R. (2003). Influence of life stress on depression: moderation by a polymorphism in the 5-HTT gene. *Science (New York, N.Y.)*, *301*(5631), 386–389. https://doi.org/10.1126/science.1083968

Cosgrove, D., Mothersill, O., Kendall, K., Konte, B., Harold, D., Giegling, I., … Donohoe, G. (2017). Cognitive Characterization of Schizophrenia Risk Variants Involved in Synaptic Transmission: Evidence of CACNA1C's Role in Working Memory. *Neuropsychopharmacology*. https://doi.org/10.1038/npp.2017.123

Dao, D. T., Mahon, P. B., Cai, X., Kovacsics, C. E., Blackwell, R. A., Arad, M., … Gould, T. D. (2010). Mood Disorder Susceptibility Gene CACNA1C Modifies Mood-Related Behaviors in Mice and Interacts with Sex to Influence Behavior in

Mice and Diagnosis in Humans. *Biological Psychiatry*, *68*(9), 801–810. https://doi.org/10.1016/j.biopsych.2010.06.019

Dedic, N., Pöhlmann, M. L., Richter, J. S., Mehta, D., Czamara, D., Metzger, M. W., … Deussing, J. M. (2017). Cross-disorder risk gene CACNA1C differentially modulates susceptibility to psychiatric disorders during development and adulthood. *Molecular Psychiatry*. https://doi.org/10.1038/mp.2017.133

Dietsche, B., Backes, H., Laneri, D., Weikert, T., Witt, S. H., Rietschel, M., … Krug, A. (2014). The impact of a CACNA1C gene polymorphism on learning and hippocampal formation in healthy individuals: A diffusion tensor imaging study. *NeuroImage*, *89*, 256–261. https://doi.org/10.1016/j.neuroimage.2013.11.030

Dutta, S., & Sengupta, P. (2016). Men and mice: Relating their ages. *Life Sciences*, *152*, 244–248. https://doi.org/10.1016/j.lfs.2015.10.025

Eckart, N., Song, Q., Yang, R., Wang, R., Zhu, H., McCallion, A. S., & Avramopoulos, D. (2016). Functional Characterization of Schizophrenia-Associated Variation in CACNA1C. *PLOS ONE*, *11*(6), e0157086. https://doi.org/10.1371/journal.pone.0157086

Ellenbroek, B., & Youn, J. (2016). Rodent models in neuroscience research: is it a rat race? *Disease Models & Mechanisms*, *9*(10), 1079–1087. https://doi.org/10.1242/dmm.026120

Erk, S., Meyer-Lindenberg, A., Schnell, K., von Boberfeld, C. O., Esslinger, C., Kirsch, P., … others. (2010). Brain function in carriers of a genome-wide supported bipolar disorder variant. *Archives of General Psychiatry*, *67*(8), 803–811.

Ferreira, M. A. R., O'Donovan, M. C., Meng, Y. A., Jones, I. R., Ruderfer, D. M., Jones, L., … Craddock, N. (2008). Collaborative genome-wide association analysis supports a role for ANK3 and CACNA1C in bipolar disorder. *Nature Genetics*, *40*(9), 1056–1058. https://doi.org/10.1038/ng.209

Gershon, E. S., Grennan, K., Busnello, J., Badner, J. A., Ovsiew, F., Memon, S., … Liu, C. (2014). A rare mutation of CACNA1C in a patient with bipolar disorder, and decreased gene expression associated with a bipolar-associated common SNP of CACNA1C in brain. *Molecular Psychiatry*, *19*(8), 890–894. https://doi.org/10.1038/mp.2013.107

Geurts, A. M., Cost, G. J., Freyvert, Y., Zeitler, B., Miller, J. C., Choi, V. M., … Buelow, R. (2009). Knockout Rats via Embryo Microinjection of Zinc-Finger Nucleases. *Science*, *325*(5939), 433–433. https://doi.org/10.1126/science.1172447

Goldberg, J. F., & Chengappa, K. N. R. (2009). Identifying and treating cognitive impairment in bipolar disorder. *Bipolar Disorders*, *11*(s2), 123–137.

Görisch, J., & Schwarting, R. (2006). Wistar rats with high versus low rearing activity differ in radial maze performance. *Neurobiology of Learning and Memory*, *86*(2), 175–187. https://doi.org/10.1016/j.nlm.2006.02.004

Green, E. K., Grozeva, D., Jones, I., Jones, L., Kirov, G., Caesar, S., … others. (2010). The bipolar disorder risk allele at CACNA1C also confers risk of recurrent major depression and of schizophrenia. *Molecular Psychiatry*, *15*(10), 1016.

Hammar, A., Lund, A. & Hugdahl, K. (2003). Selective impairment in effortful information processing in major depression. *Journal of the International Neuropsychological Society*, *9*(6), 954-959. https://doi.org/10.1017/S1355617703960152

Harmer, C. J., O'Sullivan, U., Favaron, E., Massey-Chase, R., Ayres, R., Reinecke, A., … Cowen, P. J. (2009). Effect of acute antidepressant administration on negative affective bias in depressed patients. *American Journal of Psychiatry*, *166*(10), 1178–1184.

Homberg, J. R., Wöhr, M., & Alenina, N. (2017). Comeback of the Rat in Biomedical Research. *ACS Chemical Neuroscience*, *8*(5), 900–903. https://doi.org/10.1021/acschemneuro.6b00415

Jeon, D., Kim, S., Chetana, M., Jo, D., Ruley, H. E., Lin, S.-Y., … Shin, H.-S. (2010). Observational fear learning involves affective pain system and Cav1.2 Ca2+ channels in ACC. *Nature Neuroscience*, *13*(4), 482–488. https://doi.org/10.1038/nn.2504

Kabir, Z. D., Lee, A. S., Burgdorf, C. E., Fischer, D. K., Rajadhyaksha, A. M., Mok, E., … Rajadhyaksha, A. M. (2016). Cacna1c in the Prefrontal Cortex Regulates Depression-Related Behaviors via REDD1. *Neuropsychopharmacology*, *42*, 2032.

Kabir, Z. D., Lee, A. S., & Rajadhyaksha, A. M. (2016). L-type Ca2+ channels in mood, cognition and addiction: integrating human and rodent studies with a focus on behavioural endophenotypes: L-type Ca2+ channels in mood, cognition and addiction. *The Journal of Physiology*, *594*(20), 5823–5837. https://doi.org/10.1113/JP270673

Kabitzke, P. A., Brunner, D., He, D., Fazio, P. A., Cox, K., Sutphen, J., … Clayton, A. L. (2017). Comprehensive analysis of two Shank3 and the Cacna1c mouse models of autism spectrum disorder: Shank3 and Cacna1c Models of ASD. *Genes, Brain and Behavior*. https://doi.org/10.1111/gbb.12405

Kaufman, J., Yang, B.-Z., Douglas-Palumberi, H., Grasso, D., Lipschitz, D., Houshyar, S., … Gelernter, J. (2006). Brain-derived neurotrophic factor-5-HTTLPR gene interactions and environmental modifiers of depression in children. *Biological Psychiatry*, *59*(8), 673–680. https://doi.org/10.1016/j.biopsych.2005.10.026

Kempton, M.J., Ruberto, G., Vassos, E., Tatarelli, R., Girardi, P., Collier, D., Frangou, S. (2009). Effects of the CACNA1C risk allele for bipolar disorder on cerebral gray matter volume in healthy individuals. American Journal of Psychiatry, 166(12):1413-1414. https//doi: 10.1176/appi.ajp.2009.09050680.

Kendler, K. S., Myers, J., & Prescott, C. A. (2005). Sex differences in the relationship between social support and risk for major depression: a longitudinal study of opposite-sex twin pairs. *The American Journal of Psychiatry*, *162*(2), 250–256. https://doi.org/10.1176/appi.ajp.162.2.250

Kessler, R. C., Berglund, P., Demler, O., Jin, R., Merikangas, K. R., & Walters, E. E. (2005). Lifetime prevalence and age-of-onset distributions of DSM-IV disorders in the National Comorbidity Survey Replication. *Archives of General Psychiatry*, *62*(6), 593–602.

Krug, A., Nieratschker, V., Markov, V., Krach, S., Jansen, A., Zerres, K., … Kircher, T. (2010). Effect of CACNA1C rs1006737 on neural correlates of verbal fluency in healthy individuals. *NeuroImage*, *49*(2), 1831–1836. https://doi.org/10.1016/j.neuroimage.2009.09.028

Krug, A., Witt, S.H., Backes, H., Dietsche, B., Nieratschker, V., Shah, N.J., Nöthen, M.M., Rietschel, M. & Kircher, T. (2014) A genome-wide supported variant in CACNA1C influences hippocampal activation during episodic memory encoding

and retrieval. *European Archives of Psychiatry and Clinical Neurosciences*, *264*(2):103-110. https://doi.org/10.1007/s00406-013-0428-x

Kurtz, M. M., & Gerraty, R. T. (2009). A meta-analytic investigation of neurocognitive deficits in bipolar illness: Profile and effects of clinical state. *Neuropsychology*, *23*(5), 551–562. https://doi.org/10.1037/a0016277

Lancaster, T. M., Heerey, E. A., Mantripragada, K., & Linden, D. E. J. (2014). CACNA1C risk variant affects reward responsiveness in healthy individuals. *Translational Psychiatry*, *4*(10), e461. https://doi.org/10.1038/tp.2014.100

McKinney, B. C., Sze, W., White, J. A., & Murphy, G. G. (2008). L-type voltage-gated calcium channels in conditioned fear: A genetic and pharmacological analysis. *Learning & Memory*, *15*(5), 326–334. https://doi.org/10.1101/lm.893808

Millan, M. J., Agid, Y., Brüne, M., Bullmore, E. T., Carter, C. S., Clayton, N. S., … Young, L. J. (2012). Cognitive dysfunction in psychiatric disorders: characteristics, causes and the quest for improved therapy. *Nature Reviews Drug Discovery*, *11*(2), 141–168. https://doi.org/10.1038/nrd3628

Moosmang, S. (2005). Role of Hippocampal Cav1.2 Ca2+ Channels in NMDA Receptor-Independent Synaptic Plasticity and Spatial Memory. *Journal of Neuroscience*, *25*(43), 9883–9892. https://doi.org/10.1523/JNEUROSCI.1531-05.2005

Morellini, F. (2013). Spatial memory tasks in rodents: what do they model? *Cell and Tissue Research*, *354*(1), 273–286. https://doi.org/10.1007/s00441-013-1668-9

Nyegaard, M., Demontis, D., Foldager, L., Hedemand, A., Flint, T. J., Sørensen, K. M., … Børglum, A. D. (2010). CACNA1C (rs1006737) is associated with schizophrenia. *Molecular Psychiatry*, *15*(2), 119–121. https://doi.org/10.1038/mp.2009.69

Paulus, F. M., Bedenbender, J., Krach, S., Pyka, M., Krug, A., Sommer, J., … Jansen, A. (2014). Association of rs1006737 in CACNA1C with alterations in prefrontal activation and fronto-hippocampal connectivity: CACNA1C rs1006737 Effects on Prefrontal Functioning. *Human Brain Mapping*, *35*(4), 1190–1200. https://doi.org/10.1002/hbm.22244

Perälä, J., Suvisaari, J., Saarni, S. I., Kuoppasalmi, K., Isometsä, E., Pirkola, S., … others. (2007). Lifetime prevalence of psychotic and bipolar I disorders in a general population. *Archives of General Psychiatry*, *64*(1), 19–28.

Perrier, E., Pompei, F., Ruberto, G., Vassos, E., Collier, D., & Frangou, S. (2011). Initial evidence for the role of CACNA1C on subcortical brain morphology in patients with bipolar disorder. *European Psychiatry*, *26*(3), 135–137. https://doi.org/10.1016/j.eurpsy.2010.10.004

Ripke, S., Neale, B. M., Corvin, A., Walters, J. T. R., Farh, K.-H., Holmans, P. A., … O'Donovan, M. C. (2014). Biological insights from 108 schizophrenia-associated genetic loci. *Nature*, *511*(7510), 421–427. https://doi.org/10.1038/nature13595

Rolstad, S., Sellgren Majkowitz, C., Joas, E., Ekman, C. J., Pålsson, E., & Landén, M. (2016). Polymorphisms of BDNF and CACNA1C are not associated with cognitive functioning in bipolar disorder or healthy controls. *Cognitive Neuropsychiatry*, *21*(3), 271–278. https://doi.org/10.1080/13546805.2016.1185405

Roussos, P., Giakoumaki, S. G., Georgakopoulos, A., Robakis, N. K., & Bitsios, P. (2011). The CACNA1C and ANK3 risk alleles impact on affective personality traits and startle reactivity but not on cognition or gating in healthy males: CACNA1C and ANK3 polymorphisms, and endophenotypes. *Bipolar Disorders*, *13*(3), 250–259. https://doi.org/10.1111/j.1399-5618.2011.00924.x

Sittig, L. J., Carbonetto, P., Engel, K. A., Krauss, K. S., Barrios-Camacho, C. M., & Palmer, A. A. (2016). Genetic Background Limits Generalizability of Genotype-Phenotype Relationships. *Neuron*, *91*(6), 1253–1259. https://doi.org/10.1016/j.neuron.2016.08.013

Sklar, P., Smoller, J. W., Fan, J., Ferreira, M. a. R., Perlis, R. H., Chambert, K., … Purcell, S. M. (2008). Whole-genome association study of bipolar disorder. *Molecular Psychiatry*, *13*(6), 558–569. https://doi.org/10.1038/sj.mp.4002151

Soeiro-de-Souza, M. G., Bio, D. S., Dias, V. V., Vieta, E., Machado-Vieira, R., & Moreno, R. A. (2013). The CACNA1C risk allele selectively impacts on executive function in bipolar type I disorder. *Acta Psychiatrica Scandinavica*, *128*(5), 362–369. https://doi.org/10.1111/acps.12073

Temme, S. J., Bell, R. Z., Fisher, G. L., & Murphy, G. G. (2016). Deletion of the Mouse Homolog of CACNA1C Disrupts Discrete Forms of Hippocampal-Dependent Memory and Neurogenesis within the Dentate Gyrus. *ENeuro*, *3*(6). https://doi.org/10.1523/ENEURO.0118-16.2016

Valluy, J., Bicker, S., Aksoy-Aksel, A., Lackinger, M., Sumer, S., Fiore, R., … Schratt, G. (2015). A coding-independent function of an alternative Ube3a transcript during neuronal development. *Nature Neuroscience*, *18*(5), 666–673. https://doi.org/10.1038/nn.3996

White, J. A., McKinney, B. C., John, M. C., Powers, P. A., Kamp, T. J., & Murphy, G. G. (2008). Conditional forebrain deletion of the L-type calcium channel CaV1.2 disrupts remote spatial memories in mice. *Learning & Memory*, *15*(1), 1–5. https://doi.org/10.1101/lm.773208

Widom, C. S., DuMont, K., & Czaja, S. J. (2007). A prospective investigation of major depressive disorder and comorbidity in abused and neglected children grown up. *Archives of General Psychiatry*, *64*(1), 49–56. https://doi.org/10.1001/archpsyc.64.1.49

Yoshimizu, T., Pan, J. Q., Mungenast, A. E., Madison, J. M., Su, S., Ketterman, J., … Tsai, L.-H. (2015). Functional implications of a psychiatric risk variant within CACNA1C in induced human neurons. *Molecular Psychiatry*, *20*(2), 162–169. https://doi.org/10.1038/mp.2014.143

Zanos, P., Bhat, S., Terrillion, C. E., Smith, R. J., Tonelli, L. H., & Gould, T. D. (2015). Sex-dependent modulation of age-related cognitive decline by the L-type calcium channel gene Cacna1c. *European Journal of Neuroscience*, *42*(8), 2499–2507. https://doi.org/10.1111/ejn.12952

Zhang, Q., Shen, Q., Xu, Z., Chen, M., Cheng, L., Zhai, J., … others. (2012). The effects of CACNA1C gene polymorphism on spatial working memory in both healthy controls and patients with schizophrenia or bipolar disorder. *Neuropsychopharmacology*, *37*(3), 677.

FIGURES

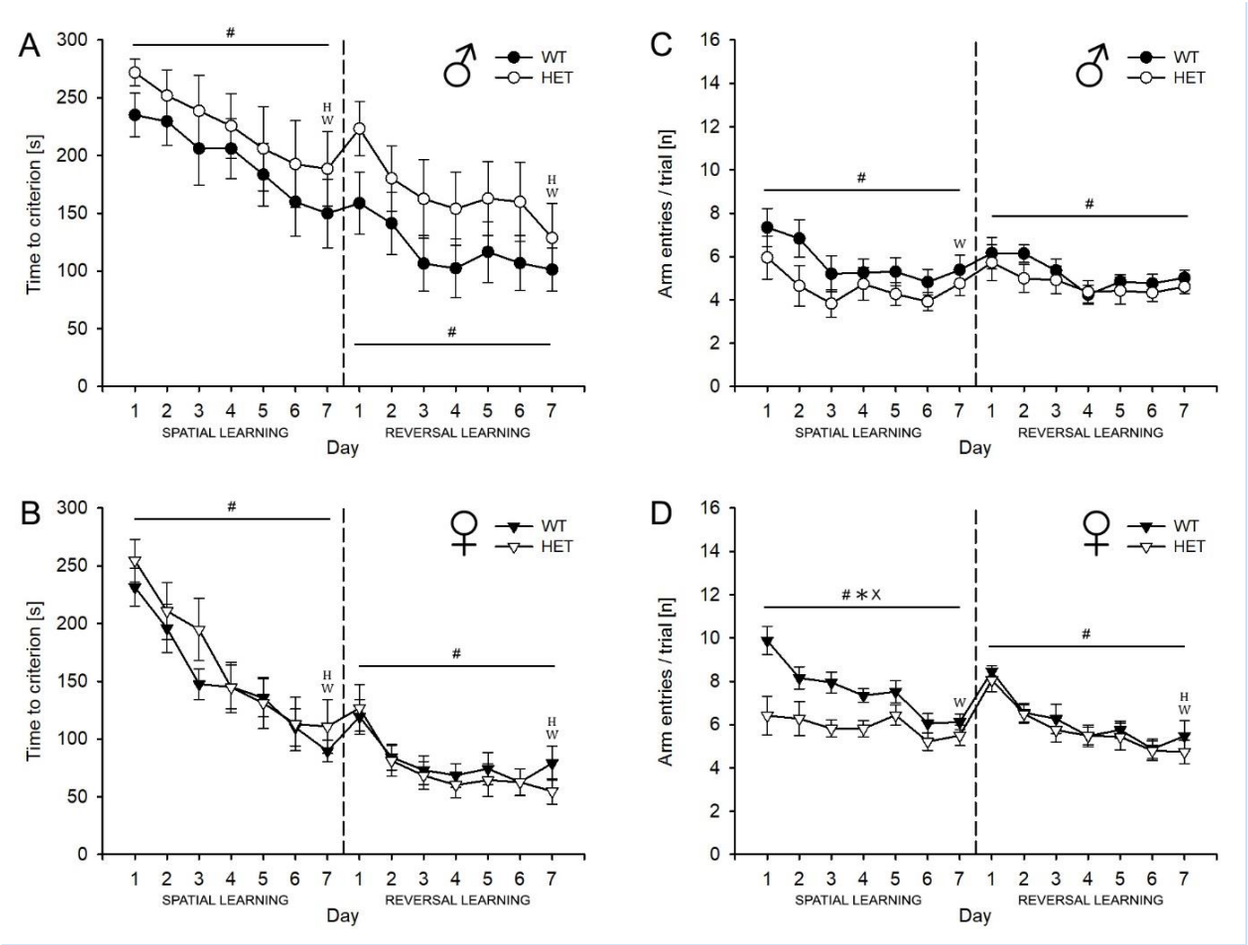

Figure 1. All experimental groups required less time and fewer entries across training days, in females with initial hypo-activity in heterozygous *Cacna1c*$^{+/-}$ and a steeper decline in wildtype *Cacna1c*$^{+/+}$ during spatial learning. (a)(b) Average latency during spatial (left part) and reversal learning (right part) to collect all 8 pellets per day in *Cacna1c*$^{+/+}$ and *Cacna1c*$^{+/-}$ depicted for males (a) and females (b). All experimental groups became faster over days irrespective of genotype or learning phase. (c)(d) Average number of arm entries per trial until all pellets were found in *Cacna1c*$^{+/+}$ and *Cacna1c*$^{+/-}$ depicted for males (c) and females (d). All experimental groups reduced the number of entries over time. During spatial learning, *Cacna1c*$^{+/-}$ females displayed initial hypo-activity, while *Cacna1c*$^{+/+}$ females started off with more entries and reduced more drastically over time, resulting in a significant decrease from day 1 to day 7. The dashed line represents the switch from spatial to reversal learning. Time cut-off criterion was 300s. Data are presented as means (±SEM). #=p<0.05 (main effect training day). *=p<0.05 (main effect genotype). x=p<0.05 (interaction day x genotype). W=p<0.05 (comparison day 1 and 7 in *Cacna1c*$^{+/+}$). H=p<0.05 (comparison day 1 and 7 in *Cacna1c*$^{+/-}$).

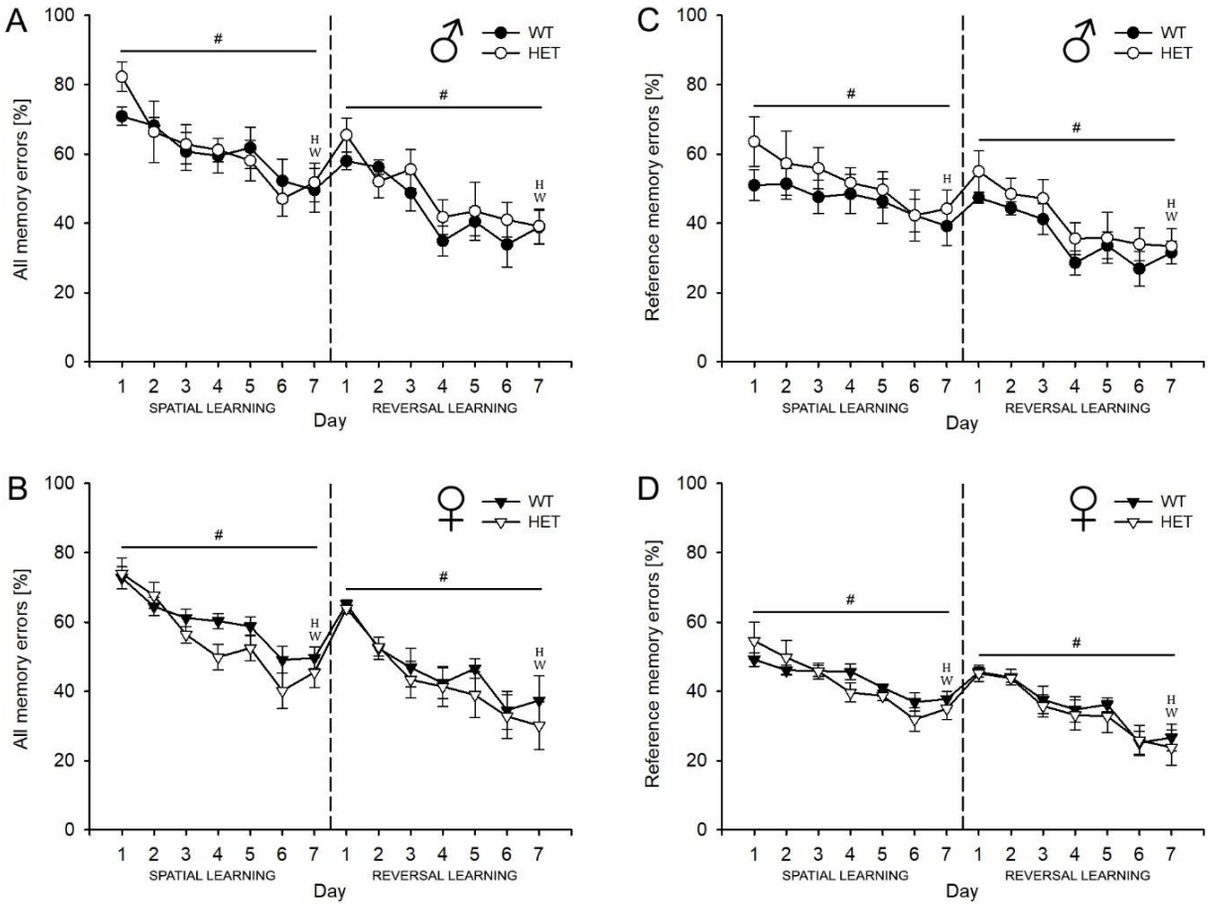

Figure 2. Intact spatial and reversal learning abilities in all experimental groups. (a)(b) Percentage of all wrong arm entries relative to total number of entries in wildtype *Cacna1c*$^{+/+}$ and *Cacna1c*$^{+/-}$ depicted for males (a) and females (b). All experimental groups reduced their total error percentages over time. (c)(d) Percentage of RM errors, initial entries into unrewarded arms relative to total number of entries, in *Cacna1c*$^{+/+}$ and *Cacna1c*$^{+/-}$ depicted for males (c) and females (d). All experimental groups reduced the percentage of reference memory errors over time. In males, only *Cacna1c*$^{+/-}$ showed a significant reduction during spatial learning, whereas *Cacna1c*$^{+/+}$ started off with fewer errors and remained that way. The dashed line represents the switch from spatial to reversal learning. Data are presented as means (±SEM). #=p<0.05 (main effect training day). W=p<0.05 (comparison day 1 and 7 in *Cacna1c*$^{+/+}$). H=p<0.05 (comparison day 1 and 7 in *Cacna1c*$^{+/-}$).

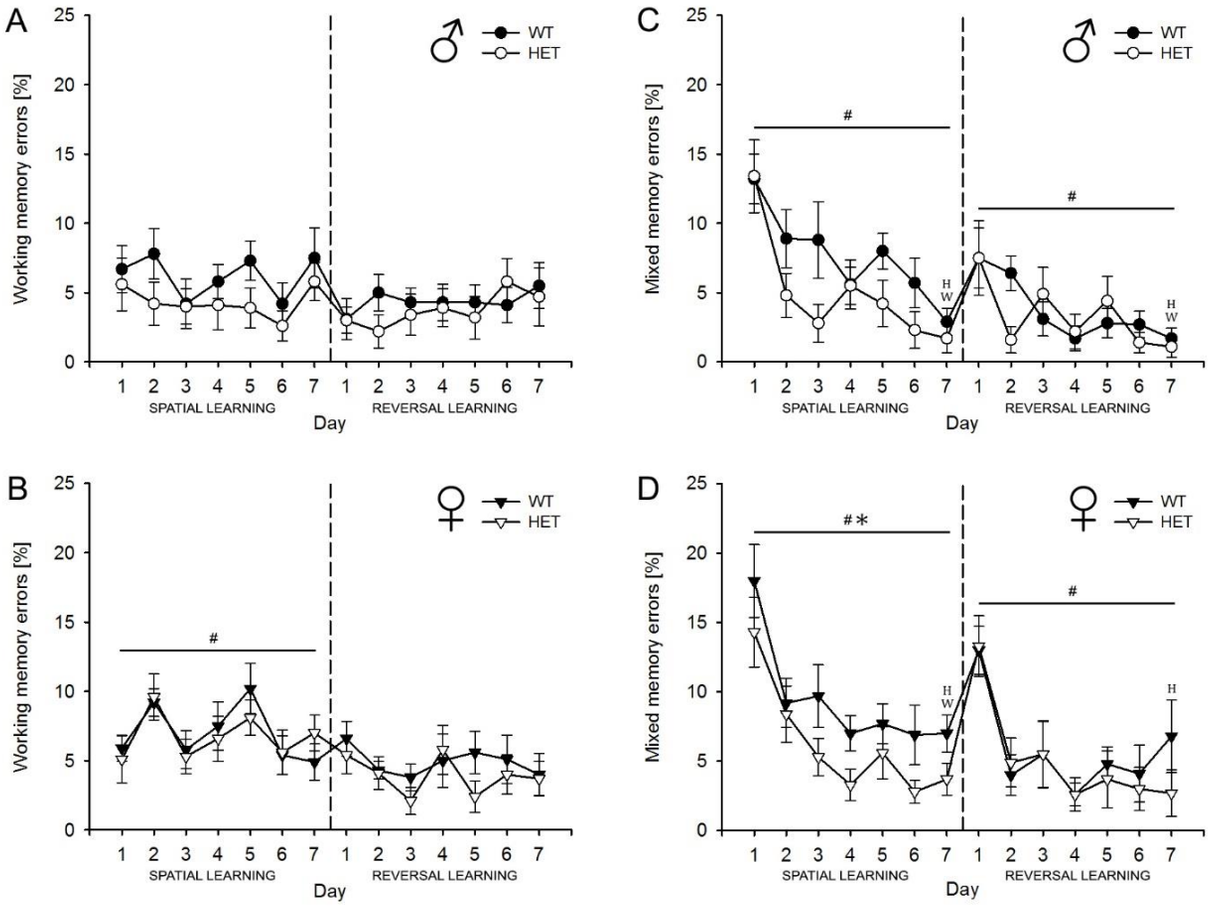

Figure 3. No improvement in working memory performance, but declining mixed errors with better performance in heterozygous *Cacna1c*$^{+/-}$ females. (a)(b) Percentage of repeated entries into *rewarded* arms relative to total number of entries in wildtype *Cacna1c*$^{+/+}$ and *Cacna1c*$^{+/-}$ depicted for males (a) and females (b). No significant improvement in WM errors was achieved. (c)(d) Percentage of repeated entries into *unrewarded* arms relative to total number of entries in *Cacna1c*$^{+/+}$ and *Cacna1c*$^{+/-}$ depicted for males (c) and females (d). All experimental groups reduced their percentage of MIX memory errors across training days, with a lower error percentage in *Cacna1c*$^{+/-}$ females during spatial learning and no improvement from day 1 to day 7 in *Cacna1c*$^{+/+}$ females during reversal. The dashed line represents the switch to reversal learning. Data are presented as means (±SEM). #=p<0.05 (main effect training day). *=p<0.05 (main effect genotype). W=p<0.05 (comparison day 1 and 7 in *Cacna1c*$^{+/+}$). H=p<0.05 (comparison day 1 and 7 in *Cacna1c*$^{+/-}$).

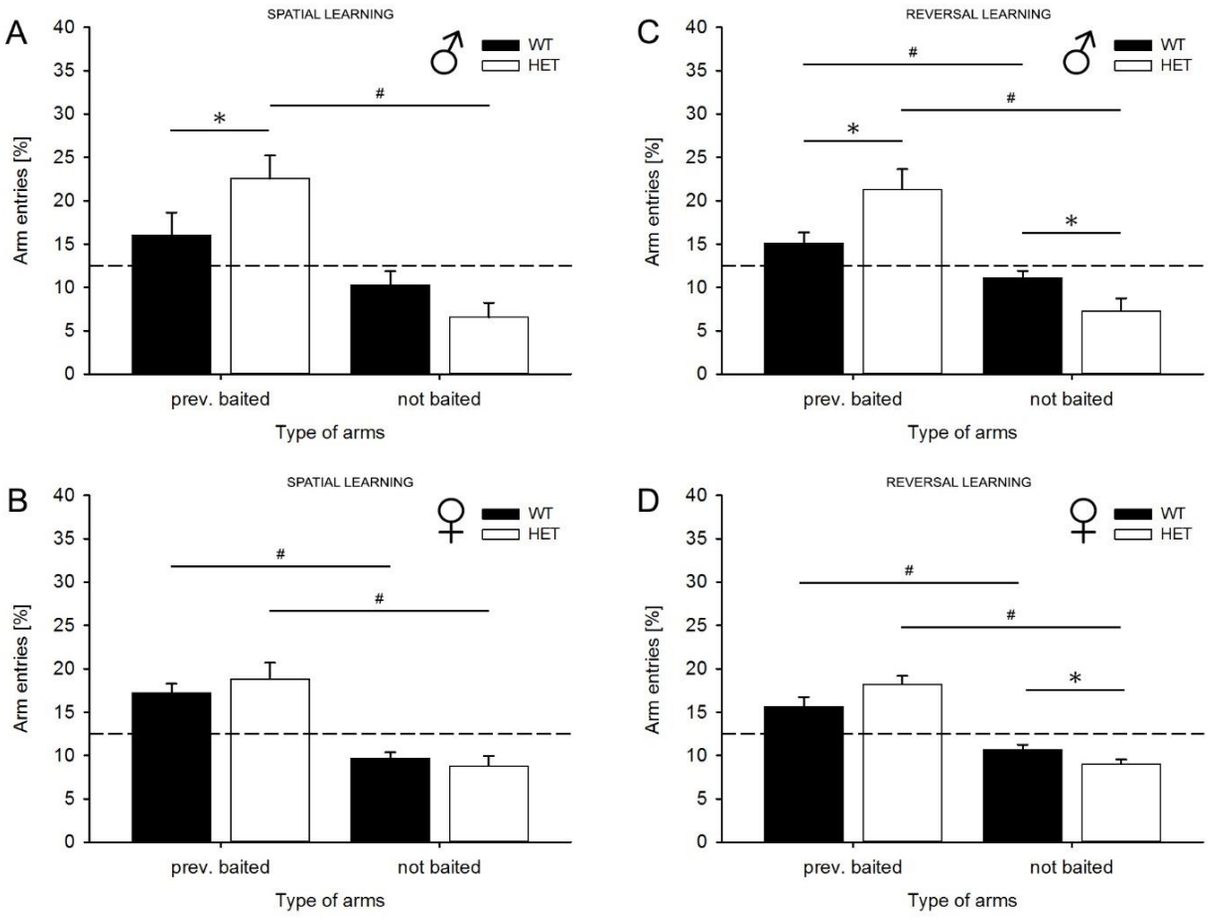

Figure 4. Intact memory retention during probe trials with stronger memory traces in heterozygous *Cacna1c*$^{+/-}$ males. (a)(b) Percentage of arm entries into previously baited arms during spatial learning relative to all probe entries and adjusted for number of arms of the specific type in wildtype *Cacna1c*$^{+/+}$ and heterozygous *Cacna1c*$^{+/-}$ depicted for males (a) and females (b). All experimental groups except for *Cacna1c*$^{+/+}$ males preferred the previously baited arms during spatial learning probe. *Cacna1c*$^{+/-}$ males showed a stronger preference for the previously baited arms than their siblings. (c)(d) Percentage of arm entries into previously baited arms during reversal learning relative to all probe entries in *Cacna1c*$^{+/+}$ and *Cacna1c*$^{+/-}$ depicted for males (c) and females (d). All experimental groups preferred the previously baited arms during reversal learning probe. Preference for baited arms was higher in *Cacna1c*$^{+/-}$ males than in *Cacna1c*$^{+/+}$, and vice versa for non-baited arms. The dashed line represents chance level. Data are presented as means (±SEM). #=p<0.05 (within genotype). *=p<0.05 (between genotypes).

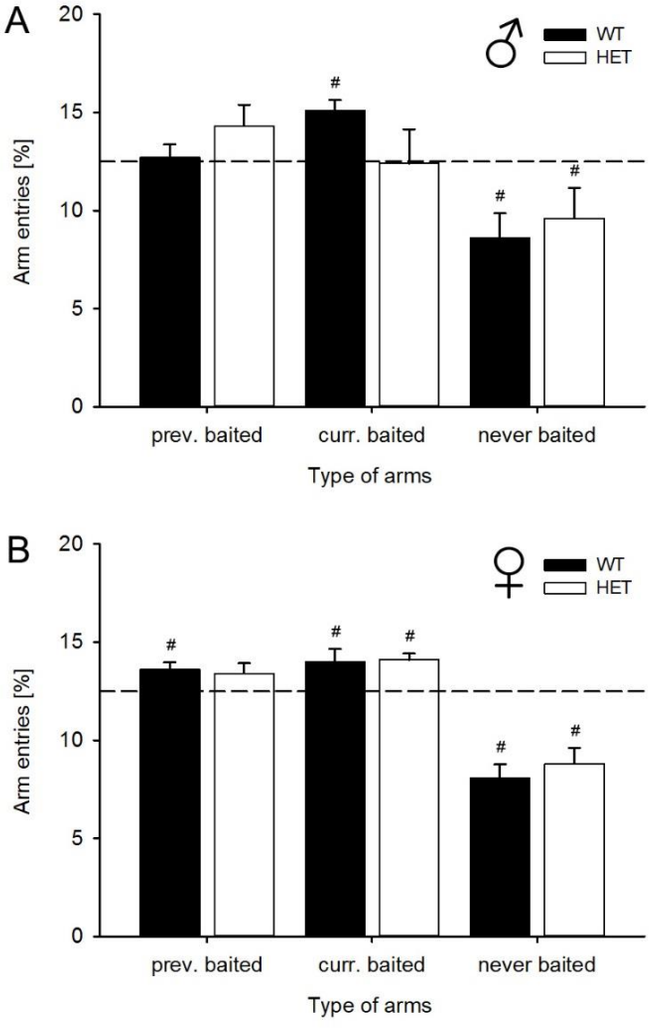

Figure 5. Intact cognitive flexibility on reversal learning day 1 in wildtype *Cacna1c*$^{+/+}$ males and heterozygous *Cacna1c*$^{+/-}$ females, with perseveration tendencies in *Cacna1c*$^{+/-}$ males and *Cacna1c*$^{+/+}$ females. (a)(b) Percentage of entries made into previously rewarded arms from spatial learning, currently rewarded arms from this day forward and the two never baited arms in *Cacna1c*$^{+/+}$ and *Cacna1c*$^{+/-}$ depicted for males (a) and females (b). Male *Cacna1c*$^{+/+}$ already show preference for the new arms, while *Cacna1c*$^{+/-}$ males do not. In females *Cacna1c*$^{+/+}$ seek out both previously and currently rewarded arms in an above chance frequency while *Cacna1c*$^{+/-}$ only show a preference for the newly rewarded arm. The dashed line represents chance level. Data are presented as means (±SEM). #=p<0.05 (within genotype, comparison to chance level).

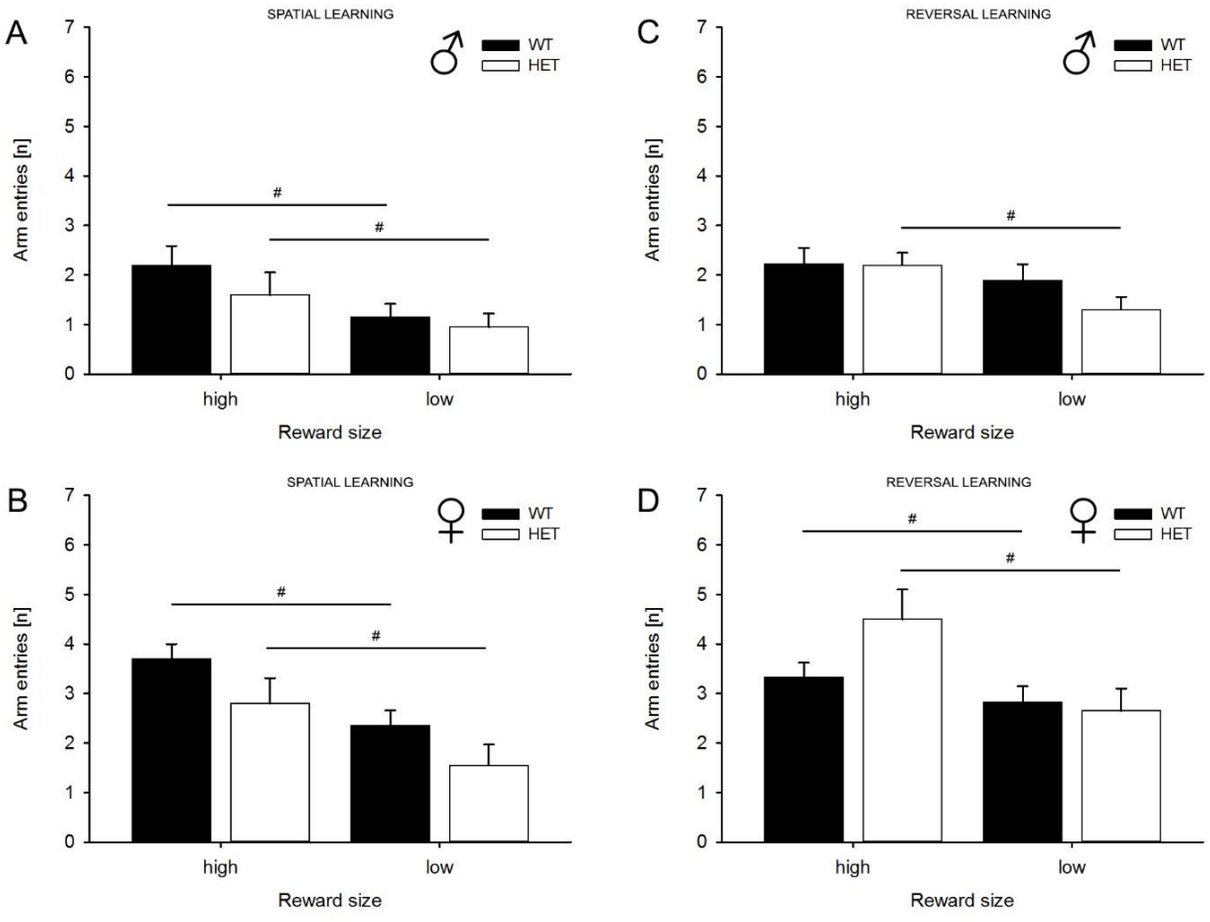

Figure 6. Intact reward sensitivity in wildtype *Cacna1c*$^{+/+}$ and heterozygous *Cacna1c*$^{+/-}$ as assessed during probe trials. Number of entries made into the arm previously containing six pellets (high) and arms with one pellet during learning (low, average of 2 arms) during spatial learning probe trial (a)(b) and reversal learning probe trial (c)(d) in *Cacna1c*$^{+/+}$ and *Cacna1c*$^{+/-}$ depicted for males (a)(c) and females (b)(d). All experimental groups preferred the higher rewarded arm over the lower rewarded arms during the spatial learning probe trial. In the reversal learning probe trial, male *Cacna1c*$^{+/+}$ no longer showed a preference (c). The dashed line represents chance level. Data are presented as means (±SEM). #=p<0.05 (within genotype).

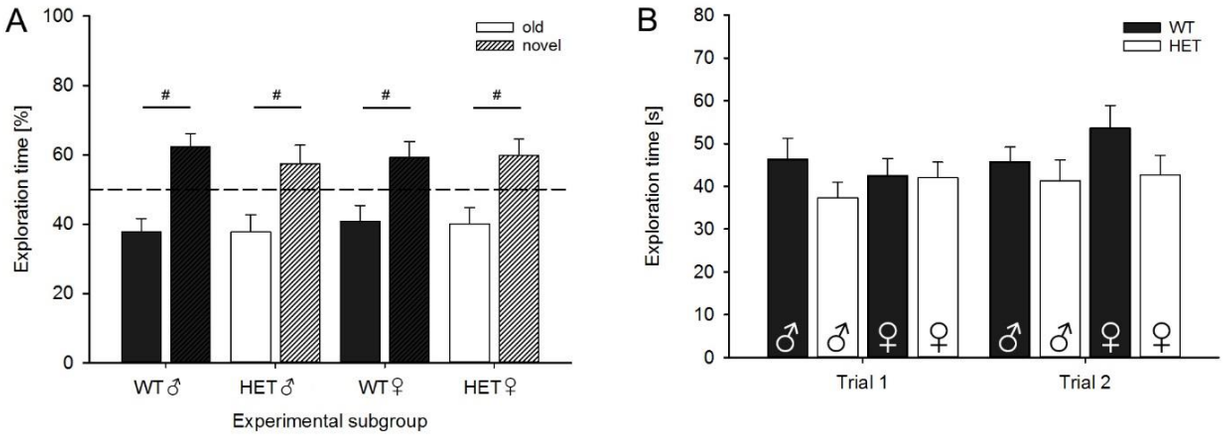

Figure 7. Intact novel object recognition in wildtype *Cacna1c*$^{+/+}$ *and heterozygous Cacna1c*$^{+/-}$. (a) Depicted is the time spent sniffing (percentage of total exploration in trial 2) the novel object (patterned bars) vs. the familiar object (blank bars). All experimental groups could distinguish between novel and old object, preferring to sniff the novel object for longer. (b) Total exploration of both objects in both trials. There was no difference between *Cacna1c*$^{+/+}$ and *Cacna1c*$^{+/-}$ in seconds spent exploring in trial 1 or trial 2 in either sex. The dashed line represents chance level. Data are presented as means (±SEM). *=p<0.05.

General Discussion

GWAS have identified several risk genes implicated neuropsychiatric disorders, recently *CACNA1C* has emerged as a prime candidate susceptibility gene linked to affective disorders, MDD and BPD as well as, neurodevelopmental disorders SCZ and ASD (Bhat et al., 2012; Heyes, et al., 2015; Ou et al., 2015). Several studies have linked mutations in the *CACNA1C* gene, specifically G406R, to ASD and ASD-like disorders, such as TS. The *CACNA1C* mutation in TS results in a gain of function, leading to an attenuation of the voltage-dependent inhibition of the $Ca_v1.2$ channel (Barrett & Tsien, 2008). In relation to neuropsychiatric disorders, studies have found both a loss and a gain of function in post mortem human brain tissue in patients with SCZ and BPD (Bigos et al., 2010; Gershon et al., 2014; Roussos et al., 2014; Yoshimizu et al., 2015). Moreover, in early research on Ca^{2+} it was reported that patients with BPD experiencing manic episodes had decreased Ca^{2+} levels (Carman & Wyatt, 1979), while BPD patients experiencing depressive episodes had elevated Ca^{2+} levels (Jimerson, Post, Carman, et al., 1979; Levine, Stein, Rapoport, & Kurtzman, 1999) and further studies later confirmed elevated Ca^{2+} levels in BPD patients (Dubovsky, Thomas, Hijazi, & Murphy, 1994; Emamghoreishi, Schlichter, Li, et al., 1997). Unsurprisingly, this led to the hypothesis that a dysregulation in Ca^{2+} levels could explain neuropsychiatric disorders such as BPD, for example. Naturally, however, the explanations are much more complicated.

The well-known *CACNA1C* risk allele rs1006737 has been highly connected to several major neuropsychiatric disorders, such as MDD, BPD, and SCZ, in behavioral and structural brain alterations (for comprehensive review see: (Berger & Bartsch, 2014; Kabir et al., 2016)). Interestingly, social behavior and communication deficits, as well as cognitive impairments are key characteristics of four major neuropsychiatric disorders linked to *CACNA1C*, suggesting that the *CACNA1C* gene may have a potential role in regulating social behavior and social communication. Humans are a naturally gregarious species, not unlike the rat, and much of our daily lives rely on social interactions, communication with peers and adept cognitive abilities. Thus, when proper social or cognitive functioning is impaired an individual's day to day life can sometimes become too much, and it can lead to severe depression and even suicide (Lépine & Briley, 2011). Evidence provided by *Cacna1c* mouse models has suggested that $Ca_v1.2$ channel malfunctioning can result in social behavior impairments (Bader et al., 2011;

Kabitzke, Brunner, He, et al., 2017) as well as cognitive deficits (Kabir et al., 2016). Importantly, key studies in which social impairments are observed are using a mouse model in which $Ca_v1.2$ expression is enhanced (Bader et al., 2011; Kabitzke et al., 2017). Conversely, very little is known about the effects of decreased $Ca_v1.2$ channel expression on social behavior or communication, until now. The purpose of this thesis was to further explore the functional role the *Cacna1c* gene has on social behavior and communication as well as cognitive functioning in a newly-developed haploinsufficient *Cacna1c* rat model in which one copy of the *Cacna1c* gene is deleted.

By means of two comprehensive review papers, the importance of two central methods utilized in the current dissertation, specifically, juvenile rough-and-tumble play and 50-kHz USV emission (Review I) and pro-social 50-kHz USV playback (Review II), are discussed and reasoned to be effective approaches for investigating changes in social interactions during the critical period of development in rats in both the senders and receivers. Subsequently, Study I and II takes advantage of the two central methods employed in Review I and Review II to empirically investigate the role of globally decreased $Ca_v1.2$ expression in male and female rats, during the critical period of development after weaning. In humans GWAS have suggested that the *CACNA1C* risk allele rs1006737 is associated with cognitive impairments (Kabir et al., 2016) and with this in mind, and based on evidence from *Cacna1c* mouse models (Temme et al., 2016; J. A. White et al., 2008), Study III investigates the role global *Cacna1c* haploinsufficiency may have on cognitive functions in adult male and female *Cacna1c* rats. Taken together, the information provided through Review I and II and empirical Study I, II, and III composing this dissertation suggest that (i) juvenile social behavior and 50-kHz USV are important measures for detecting behavior and communication impairments reminiscent of neuropsychiatric disorders and (ii) *Cacna1c* haploinsufficiency in rats leads to sex-specific differences in social play and ultrasonic communication as juveniles, as well as, (iii) superior spatial learning but reduced cognitive flexibility during reversal learning in adulthood.

In the following sections, I will firstly emphasize the practicality and effectiveness of the two primary behavioral approaches used, specifically rough-and-tumble play together with concomitant emission of 50-kHz USV and pro-social 50-kHz USV playback, with a specific focus on their importance for the use in empirical Study I and II. Through these two paradigms, early social behavior and communication impairments can be assessed with added value

provided by means of pro-social 50-kHz USV. Hence, next, I will focus on how a decrease in $Ca_v1.2$ expression levels lead to social communication impairments and sex-specific differences in social play and 50-kHz USV playback responses in haploinsufficient *Cacna1c* rats. To provide awareness into the role *Cacna1c* plays in the general pathophysiology of neuropsychiatric disorders, I will then discuss cognitive impairments observed in *Cacna1c* haploinsufficient adult rats and again, briefly touch on why possible sex-specific differences may emerge. Lastly, I will discuss the relevance to translational perspectives and conclude on why further research into the role of $Ca_v1.2$ channel expression using rodent models, particularly the rat, is necessary to understanding the pathophysiology of neuropsychiatric disorders in which core components are impairments in social functioning.

Social Play and 50-kHz USV: A Tool for Unlocking Emotional Underpinnings in Neuropsychiatric Disorders

For humans, psychosocial interventions have been shown to reduce levels of depression and anxiety and increase positive affective states (Lee Duckworth, Steen, & Seligman, 2005). Interestingly, positive affective states in humans and rats are elicited by the same stimuli and share homologous neuroanatomical and neurochemical underpinnings (Burgdorf & Panksepp, 2006). Eliciting a robust positive affective state in humans can be achieved simply through positive, social interactions (Csikszentmihalyi & Hunter, 2014). Likewise, in rats rewarding social interactions are repeatedly shown to be associated with increased rates of 50-kHz USV emission (Wöhr, 2018). Thus, through numerous studies it has been established that rat 50-kHz USV are a valid model for studying positive affective states (Burgdorf & Panksepp, 2006; Burgdorf et al., 2011; Panksepp & Burgdorf, 2003; but also see: Wöhr, 2018).

The ability to engage in rough-and-tumble play for juvenile rats has been shown repeatedly to be a crucial component for proper development of the brain and social behaviors needed in adulthood (Pellis & Pellis, 2009; Vanderschuren et al., 2016). Furthermore, prevention of the behavior can lead to impaired and altered behavior patterns in mating (Gruendel & Arnold, 1969; Hård & Larsson, 1968), social (Hol et al., 1999; Seffer et al., 2015) and agonistic interactions (Lore & Flannelly, 1977; C. L. Van Den Berg, van Ree, et al., 1999). More importantly, the changes observed have significant and translational relevance to neuropsychiatric disorders; which can include altered responses to drugs of abuse,

hyperactivity in a novel environment, impaired sensorimotor gating, cognitive inflexibility, and social withdrawal and impaired social communication (Fone & Porkess, 2008; Hall, 1998; Lapiz et al., 2003; Seffer et al., 2015).

One common problem consistently plaguing conspecific social interaction studies in rodents, in which USV are analyzed, is that when there are two animals interacting within the same behavioral arena, it is never certain which animal is producing the USV. Although, as shown in Review I, by using surgical manipulation, the recurrent laryngeal nerve, responsible for USV production, can be bisected which subsequently eliminates USV capabilities in the rat. Review I discusses in detail how two recent studies have revealed the essential role of 50-kHz USV within rough-and-tumble play and, additionally, demonstrated that without USV production, from either play partner, the frequency of playful interactions is severely diminished (Kisko, Euston, et al., 2015; Kisko, Himmler, et al., 2015). As was summarized in Review I, however, the playful interactions can be rescued by pairing a devocalized partner with an intact, control play partner. Moreover, upon further examination of the specific components constituting the playful repertoire, it was shown that not only does surgical devocalization diminish the motivation to engage in playful activities but it also impairs the defensive tactics chosen by the devocalized rat and that these alterations in defensive tactic are persistent whether paired with another devocalized or control play partner (Kisko, Himmler, et al., 2015). This suggests that perhaps the surgical manipulation of rats at such a young developmental age and during the critical period of play may disrupt developmental trajectories which in turn, may alter specific brain regions or connections responsible for specific behavioral tactics. In rats, dopaminergic projections in the VTA develop within the first four weeks after birth (Choong & Shen, 2004) which overlaps the peak period of play in juvenile rats; between four and five weeks (Pellis & Pellis, 2009). Production, as well as reception, of 50-kHz USV has been shown to be strongly linked to NAcc dopamine release (Burgdorf et al., 2011; Willuhn et al., 2014) suggesting that the elimination of 50-kHz USV abilities may influence the development of dopamine reward pathways and, thus, indirectly affect the motivation for play, as well as specific behaviors during play.

One essential new finding discussed in Review I, is that when two rats are engaged in pinning behaviors, the rat on the top of the pin configuration, i.e., the one doing the pinning, is producing more 50-kHz USV than the rat being pinned. Using surgical devocalization provided

the ability to specifically identify the producer of the 50-kHz USV during this important play behavior. To my knowledge this has not been done before. Pinning, is known to be a highly rewarding component of the playful repertoire (Pellis & Pellis 2009, Panksepp, 1981) and in tickling paradigms with the human hand it is thought to be mimicking the pinning interaction in conspecific partner play. Based on this, it has been hypothesized that the more the animals produce 50-kHz USV the more they seem to enjoy the experience (Burgdorf & Panksepp, 2001), and that the tickling or nuzzling of the belly of the rat being pinned is what elicits the increased production of 50-kHz USV. Therefore, this suggests that it is this specific action of tickling the belly within the sequence that is highly rewarding and in fact, Burgdorf and Panksepp (2001) showed that tickling stimulation as opposed to just light touch, "petting" stimulation creates a 352% increase in 50-kHz USV production. Indeed, this high rate of 50-kHz USV emission is the result of typical human-rat tickling experiments (Burgdorf & Panksepp, 2001; Mällo et al., 2007; Panksepp & Burgdorf, 2000, 2003; Schwarting et al., 2007). However, in Review I during conspecific partner play, we have now shown that in contrast, it is not the rat being pinned but rather the one doing the pinning that is likely gaining the most reward, thus, producing more 50-kHz USV. This, therefore, may suggest that during playful interactions in juvenile rats the goal is not to be the one getting pinned but rather, to be the one that gets to pin the other. In point of fact, during rough-and-tumble play reciprocity between the partners is essential for maintaining the playful mood and prolonging the interactions (Pellis & Pellis, 2009). Therefore, it may be that this constant back-and-forth competition to pin their opponent is what produces the high rates of 50-kHz USV. To recapitulate from Review I, however, it must be mentioned that although this precise investigation into which rat is producing 50-kHz USV during specific play behaviors is the first to be done, to my knowledge, the sample size was relatively small. Therefore, further investigation is warranted to justify the results and further elucidate the role of 50-kHz USV within specific components of rough-and-tumble play.

Importantly, Review I it was shown that that surgical manipulation does not only remove the ability to emit 50-kHz USV but also 22-kHz USV, as well, and therefore not only the reward pathways may be affected but potentially also the aversive, fear pathways. One region involved in processing and regulating responses to 22-kHz USV is the amygdala (Brudzynski, 2013, 2015) which interestingly, is also linked to specific defense patterns in juvenile rough-

and-tumble play behavior (Pellis & Pellis, 2009). This proposes that the incapacity to emit 22-kHz USV during development and regulation of social behaviors could possibly affect development of the amygdala, and its subsequent connections. Excitotoxic lesions or damage to the amygdala results in altered defense patterns used during rough-and-tumble play (Pellis & Pellis, 2009) and indeed, it has been suggested that the act of engaging in rough-and-tumble play helps develop and regulate amygdala-mediated behaviors (Baron-Cohen, Ring, Bullmore, et al., 2000; Lewis & Barton, 2006; Maaswinkel, Baars, Gispen, & Spruijt, 1996). Additionally, as was summarized in Review I, not only does the loss of USV decrease the overall motivation for play in devocalized pairs, but also, there appears to be an environmental effect on conspecifics who are not devocalized, but group-housed with those that are. Thus, it appears evident from Review I, that not only the ability to emit appropriate 50-kHz USV is important for maintaining and promoting playful interactions, but it also looks as if during the critical period of development, the combined use of USV and behavior in an appropriate and, likely, emotional learning context is also highly important. Notably, it was further summarized that during adulthood in rats, the ability to emit USV is essential when navigating ambiguous, unfamiliar social situations (Kisko, Euston, et al., 2015). In fact, higher rates of aggressive interactions were seen in pairs in which one rat is unable to vocalize, suggesting that not only are USV important for maintaining playful interactions as juveniles but that their use in adulthood is necessary in social interactions to prevent aggressive attacks, and possibly to function as appeasement signals. Indeed, it has been observed in resident intruder paradigms that often, 50-kHz USV are emitted by the intruder to try and appease the resident and prevent further aggressive attacks (Burgdorf et al., 2008). Moreover, Burke et al (2017) further found that the ability to emit specific subtypes of 50-kHz USV were important to de-escalating aggressive interactions in pairs of adult unfamiliar rats. Thus, taken together from Review I, as a juvenile it seems apparent that the appropriate learning context, provided by rough-and-tumble play and adequate environmental conditions, seem necessary to be able to successfully navigate ambiguous social interactions as adults. The longitudinal environmental effects on intact control rats housed with devocalized cagemates, however, has not been investigated and, therefore, evidence from Review I is not able to provide further insight as to the effects of inappropriate learning contexts for the use of USV during social interactions. However, understanding from 50-kHz USV playback as well as 22-kHz USV playback studies, outlined

in Review II, does give some corroborating evidence supporting the idea of a critical emotional and contextual learning stage for USV in rats with relevance to specific traits characteristic of neuropsychiatric disorders.

The second Review describes a comprehensive list of relevant studies in which playback of either 22-kHz or 50-kHz USV were utilized, and further it defines the results of behavioral and neuronal action in direct response to the playback stimuli. From this it seems apparent that the 50-kHz USV playback paradigm designed and outlined by Wöhr and Schwarting (2007) is an ideal method to study the developmental trajectory, relevance, and typical response for emotionally valanced stimuli, such as 50-kHz USV, in juvenile rats. Hence, the 50-kHz playback paradigm was an ideal method subsequently employed in our *Cacna1c* rats in Study I and II.

In combination with post-weaning social isolation during the rough-and-tumble play period, it has been summarized in Review II that 50-kHz USV playback elicits a robust response in juvenile rats that are group housed or experience short, i.e., 24-hour, isolation. Yet, no response is evident in juveniles exposed to long term, i.e., 4 weeks, isolation (Seffer et al., 2015). Importantly, the neural mechanisms and neural chemistry established in mediating and regulating responses to USV playback are homologous to areas of interest in both affective and neurodevelopmental disorders in humans (Brudzynski, 2013, 2015; Burgdorf & Panksepp, 2006) of which the *CACNA1C* gene has also been implicated (Bhat et al., 2012; Heyes et al., 2015; Ou et al., 2015). For example, the amygdala in humans has been linked to differential coding of affective valence in response to face stimuli with positive or negative valence (J. S. Morris, Frith, Perrett, et al., 1996), which also is evident in *CACNA1C* risk allele carriers (Pasparakis et al., 2015). In parallel, as was detailed and summarized in Review II, USV playback studies suggest that in rats a similar response to positive and negative USV takes place. Similarly, the NAcc has been shown by Willuhn et al (2014) to be responsible in regulating phasic dopamine release in response to 50-kHz USV playback, and in humans the fronto-limbic brain structures, including the NAcc, have been implicated in mood disorders, most prominently in BPD (Drevets, Price, & Furey, 2008; Price & Drevets, 2010). Additionally, a recent study by Frazier et al (2014) provided information that *CACNA1C* affects the volume of fronto-limbic structures in human rs1006737 risk allele carriers.

As described in Review II, pro-social 50-kHz USV playback, is a practical approach to assessing the specific influence of USV in the recipient which is of particular relevance to disorders in which emotionally valanced stimuli is unable to elicit response and results in apathy or a lack of motivation or "*wanting*" to engage in social interactions. The active approach and time spent near the ultrasonic speaker in the 50-kHz USV playback paradigm is a good indication of the "*wanting*" response because it seems that the rat is trying to find and establish social contact with the source of the USV emission, albeit at times this can also result in emission 22-kHz USV from the receiver, but this is likely just a result of frustration in not being able to find or gain access to the source emitting the 50-kHz USV. Therefore, an indication of apathy or amotivation in response to emotionally positive stimuli, for example 50-kHz USV, could be suggestive of an impairment in social functioning leading to social withdrawal, which is very often seen in affective and neurodevelopmental disorders such as MDD, BPD, SCZ and ASD (American Psychiatric Association, 2013).

In summary, Review I, accordingly, is imperative to the current dissertation primarily because it provides and in-depth discussion on one of the most recognized methods in behavioral research known to elicit a positive affective state and produce high rates of 50-kHz USV emission in rats, that of juvenile-rough-and-tumble play. Moreover, it emphasizes the importance of 50-kHz USV emission in the sender. Likewise, Review II provides further important insight into another highly practical approach to study social motivation behavior and ultrasonic communication in the receiver, namely 50-kHz USV playback. This is of particular importance, because of the specific application to modeling common impairments in social behavior and communication observed in humans diagnosed with affective or neurodevelopmental disorders. Additionally, both Review I and II aim to provide a compelling justification as to why these specific methods were chosen for the empirical portion of this dissertation in juvenile *Cacna1c* haploinsufficient rats, and how, when used together, they provide a truly reciprocal approach to studying social communication impairments. Hence, because of strong supporting evidence, outlined in Review I and II, rough-and-tumble play along with concomitant 50-kHz USV emission and pro-social 50-kHz USV playback are used in Study I and II to assess the role of a global decrease in $Ca_v1.2$ channel expression in a novel *Cacna1c* haploinsufficient rat model. *Cacna1c* rats were assessed during the critical period of development when the emergence of social behavior and communication impairments are

thought to model aspects of both affective and neurodevelopmental disorder such as MDD, BPD, SCZ and ASD.

Reduced $Ca_v1.2$ Expression: Deficits for 50-kHz USV in Males and Play in Females.

Rough-and-tumble play in juvenile rats is an ideal approach to assessing atypical developmental delays in social behavior and concomitant 50-kHz USV production (Review I). For this reason, both Study I and II employed this method to investigate the role of *Cacna1c* haploinsufficiency and further explore the disorder-dependent behavioral phenotypes found in neuropsychiatric disorders, during the early development of social behavior and communication skills.

The results of Study I and II clearly demonstrate that male *Cacna1c*$^{+/-}$ juveniles appear to have no behavioral impairments during rough-and-tumble play. Rather, the *Cacna1c*$^{+/-}$ males maintain and engage in playful interactions at levels comparable to wildtype littermate controls. This is somewhat in contrast to what was expected in terms of playful behavior for *Cacna1c*$^{+/-}$ rats. In all aspects of the playful repertoire male *Cacna1c*$^{+/-}$ males exhibited roughly equivalent frequencies and durations of pinning, wrestling and chasing behaviors. Moreover, the overall frequency and duration of playful interactions was comparable in the males for both genotypes and subsequently increased in duration and frequency across the test days. This increase was likely driven by an increase in pinning and wrestling durations in both *Cacna1c*$^{+/-}$ and *Cacna1c*$^{+/+}$ littermate controls. Intact playful interactions strongly indicate that, in terms of rough-and-tumble play behaviors, *Cacna1c*$^{+/-}$ males show no palpable impairments when compared to *Cacna1c*$^{+/+}$ littermate controls and, therefore, appear to be capable and motivated to engage in rough-and-tumble play. Both studies I and II further show that during a playful session, non-play social behaviors in males have an overall decrease across test days, regardless of genotype. This is likely driven by the typical increase in playful interactions over test days, suggesting that each day they are more motivated to engage in more playful interactions than the last. However, as was shown in Study II, even though there was a decrease of non-play social behavior over the duration of testing, on the second day, and to lesser extent the third day, *Cacna1c*$^{+/-}$ males spent more time in close physical contact with their partners than did the *Cacna1c*$^{+/+}$ littermate controls. This suggests that perhaps *Cacna1c*$^{+/-}$ males

required a little extra time to re-familiarize and reacquaint themselves with their play partner during the play session and possibly this could be due to impairments in social recognition memory. *Cacna1c* mouse models with a decrease in $Ca_v1.2$ expression have, to my knowledge, not been tested in any specific social recognition paradigms, such as those typically used in ASD mouse models (Silverman et al., 2010). However, in a modified three-chambered social approach assay *Cacna1c*$^{+/-}$ mice show a similar preference for the occupied, as opposed to empty corral, as the wildtype controls (Bader et al., 2011), indicating that a novel mouse is still as interesting to the *Cacna1c*$^{+/-}$ mouse as it is to the wildtype controls, suggesting that in this mouse model there is likely no impairments in social memory. Conversely, social recognition tests have been done in TS2-neo mice in which there is a gain of function in $Ca_v1.2$, although the results also showed no social memory impairments. Nevertheless, somewhat in parallel with our findings, Kabitzke et al (2017) report that during a reciprocal social interaction test *Cacna1c*$^{+/-}$ mouse pairs spent more time in close physical proximity with each other and they engaged in more nose-back contact than did wildtype pairs. Taken together, therefore, the limited social interaction data in *Cacna1c* mice suggests, to some extent that, *Cacna1c* haploinsufficiency may play a small role in social recognition, in that the time spent investigating familiar partners is enhanced. However, this limitation during social recognition seen by Kabitzke et al (2017) may not be evident through our rough-and-tumble play paradigm with *Cacna1c*$^{+/-}$ rats but, possibly, could become more prominent in a test specifically designed for social recognition.

Although there were no apparent behavioral differences in rough-and-tumble play between genotypes in male *Cacna1c* juveniles, there was a strong genotype difference in 50-kHz USV emission during the playful interactions, which supports the original hypothesis of expected 50-kHz USV deficits. Within a play session, it was plainly discernible that *Cacna1c*$^{+/-}$ males were consistently emitting fewer 50-kHz USV than their wildtype siblings, and this difference was persistent across all three days of testing. Low 50-kHz USV emission is suggestive of a blunted or decreased affective state (Burgdorf et al., 2011) which could indicate that *Cacna1c*$^{+/-}$ males are not enjoying the playful interactions as much as the wildtype males. Alternatively, the emotional value linked to the 50-kHz USV emission and subsequent behavioral outcome is not as robust in *Cacna1c*$^{+/-}$ rats and, thus, as a reflection of the diminished relationship they produce fewer 50-kHz USV. An important indication to measure

the motivation, or anticipation, to engage in rough-and-tumble play, is by measuring 50-kHz USV in the minutes preceding playful interactions (Knutson et al., 1998). In Study I, however, during this period there were no differences between the *Cacna1c*$^{+/-}$ and *Cacna1c*$^{+/+}$ males and in effect, each genotype showed a typical pattern of increased 50-kHz USV, both during anticipations as well as during playful interactions, from the first to the last play session. The increase of anticipatory 50-kHz USV indicates that both *Cacna1c*$^{+/-}$ and *Cacna1c*$^{+/+}$ males were indeed anticipating the forthcoming play reward. An increase in playful anticipation, therefore, would point to the *Cacna1c*$^{+/-}$ males being socially motivated to play, and this was certainly apparent in their increasing frequency of playful interactions, such as pinning and wrestling, across testing days. However, it is possible that the amount of reward they were subsequently gaining from the behavior was not enough to "really enjoy" the interaction and this was, consequently, expressed in the lower 50-kHz USV emission rates in *Cacna1c*$^{+/-}$ pairs. Alternatively, if there really is a possible disconnection between the emotional value or role of the 50-kHz USV and the social play behavior in male *Cacna1c*$^{+/-}$ rats then during rough-and-tumble play there would be no need to emit high numbers of 50-kHz USV because it may not add any additional value to the interactions.

In Study I, low 50-kHz USV emission in *Cacna1c*$^{+/-}$ males, might be due to several reasons; Firstly, it is possible that even though 50-kHz USV emission is low, it is unmistakeable that at least one partner is still producing 50-kHz calls, and as was shown in Review I, as long as there are still some 50-kHz USV being produced playful interactions can take place at rates comparable to controls (Kisko, Himmler, et al., 2015). Interestingly, Owren and Rendall (1997) hypothesized that the primary function of vocalizations in animals is not to convey information about the emotional state of the caller, but instead, to influence the emotional state of the receiver. Indeed, several studies have shown that the physiological and cognitive state of an animal can be altered by hearing species-specific vocalizations (Gil-da-Costa, Braun, Lopes, et al., 2004; Kuraoka & Nakamura, 2010). Therefore, *Cacna1c*$^{+/-}$ males may be able maintain the playful motivation and playful mood as long as there are still some 50-kHz USV being emitted. Secondly, it is entirely likely that the decrease in $Ca_v1.2$ expression alters other neural mechanisms involved in regulating affective states. For example, Burgdorf et al (2011) observed that injections of insulin-like growth factor I (IGFI) into the lateral ventricle increased rates of 50-kHz USV and that injections of siRNA specific to the

IGFI receptor decreased rates of 50-kHz USV. Based on these results Burgdorf et al (2011) suggests the IGFI has a functional role in the generation of positive affective states by means of 50-kHz USV production. In addition, through its action on the IGFI receptor, IGFI has been implicated in rapid neuronal changes, such as fast neuronal signaling through voltage-gated Ca^{2+} channels. One principle effect of IGFI modulation, therefore, might be the regulation of Ca^{2+}-dependent enzymes and in particular, transcription factors (Alberini, Ghirardl, Metz, & Kandel, 1994). Blair & Marshall (1997) report that rapid IGFI regulation of L-type voltage gated (as well as N-type) channels leads to a substantial increase in Ca^{2+} influx. Indeed, it has been found that in post-mortem human tissue of rs1006737 *CACNA1C* risk allele carriers mRNA is altered (Bigos et al., 2010; Gershon et al., 2014; Roussos et al., 2014). Thus, with a global decrease in $Ca_v1.2$ expression in our *Cacna1c* rat model it is conceivable that the regulation of proteins such as IGFI, for example, impairs the regulation of positive affective states and that this is, consequently, reflected in reduced 50-kHz USV emission rates in our rats. Lastly, in line with the potential for neurochemical impairments in proteins, alterations in the mesolimbic dopamine system can also affect 50-kHz USV production (Burgdorf et al., 2011). *Cacna1c* mouse models have, indeed, shown impairments in dopamine signaling (Kabir et al., 2016; Terrillion, Dao, et al., 2017) with specific associations between *Cacna1c* and NAcc-VTA dopamine regulation (Anderson, Famous, Sadri-Vakili, et al., 2008; Rajadhyaksha, Husson, Satpute, et al., 2004; X. F. Zhang, Cooper, & White, 2002). Moreover, the NAcc and VTA are part of the mesolimbic dopamine system implicated in the expression of endophenotypes of BPD, SCZ and MDD (Dichter, Damiano, & Allen, 2012; Russo & Nestler, 2013). Psychoactive substances, such as amphetamine, are often used in animals to model mania (Pereira et al., 2014). Recently, Dao et al (2010) showed that amphetamine administration in heterozygous *Cacna1c* mice attenuated the hyperlocomotion response, suggesting that lacking one copy of the *Cacna1c* gene alters mesolimbic dopamine mediated behaviors. This finding was further validated by Terrillion et al (2017) and additionally through fast-scan cyclic voltammetry the authors found that a reduction in *Cacna1c* leads to an attenuation of dopamine reuptake blockers after administration of a stimulant, indicating that *Cacna1c* critically regulates dopamine terminal function (Terrillion, Dao, et al., 2017). Dopamine is known to be heavily implicated throughout the NAcc and VTA in both 50-kHz USV and rough-and-tumble play behavior (Vanderschuren et al., 2016; Willuhn et al., 2014)

and therefore, impairments in the regulation of dopamine signaling between the two structures might also result in attenuation of 50-kHz USV during rewarding playful interactions.

An important point to note, however, is that an alteration in dopamine regulation may also impact specific rough-and-rumble play behaviors, as well as the overall playful motivation (Vanderschuren et al., 2016). And yet, as shown in Study I, in *Cacna1c*$^{+/-}$ males there was no observations of blatant impairments in any rough-and-tumble play at the level of frequency or duration of specific individual or overall behaviors. Possibly, according to Berridge (2007), this could be because dopaminergic neurotransmission plays a critical role in the incentive-motivational, but not hedonic pleasurable properties of natural rewards and drugs of abuse. Nonetheless, this would suggest, that in *Cacna1c*$^{+/-}$ male rats, an impairment in dopaminergic neurotransmission, as a result of altered $Ca_v1.2$ expression levels, results in impaired incentive-motivational properties reflected through low 50-kHz USV emission during rough-and-tumble play. The short period of isolation that all *Cacna1c* rats experienced before each play session may, potentially, have been enough in the male *Cacna1c*$^{+/-}$ rats to induce a pleasurable hedonic response strong enough to normalize playful interactions and produce 50-kHz USV, albeit at a diminished capacity to 50-kHz USV emission in *Cacna1c*$^{+/+}$ littermate controls. Notably, impairments in the incentive-motivational properties of 50-kHz USV in *Cacna1c*$^{+/-}$ males but also to a lesser extent in females, is paralleled in Study I and II via reduced social approach response during and after 50-kHz USV playback. Additionally, during the reciprocal social interaction test used by Kabitzke et al (2017), they note the heterozygous *Cacna1c* mice exhibited hyper-locomotion and, as a result, this could also contribute to an overall high frequency of playful interactions, such as what we observed in *Cacna1c*$^{+/-}$ males in Study I. Increased hyper-locomotion could hypothetically mirror the high rate of playful interactions seen in wildtype littermate controls. No evidence, however, has been found in any of our behavioral analysis, to date, to indicate that the male *Cacna1c*$^{+/-}$ rats are hyperactive.

In Study I, as a result of the strong reduction in 50-kHz USV production in *Cacna1c*$^{+/-}$ males during rough-and-tumble play, a further detailed exploratory, temporal and structural analysis of 50-kHz USV emission in male *Cacna1c*$^{+/-}$ and wildtype juveniles was performed. The extensive analysis revealed a difference in particular 50-kHz USV subtypes emitted during specific components of rough-and-tumble play between the genotypes. As far as what is known in the literature so far, specific subtypes of 50-kHz USV have not been linked with specific

rough-and-tumble play behaviors, although some attempts have been made to link them to the initiation of specific play behaviors as play signals, summarized in Review I. Typically, to my knowledge, in social play experiments that chose to record USV the assessment of 50-kHz USV is done by calculating the number of 50-kHz USV produced within the time period without focus on the specific subtypes. Normally subtypes that are FM are reported in rewarding circumstances such as mating, social play and administration of psychostimulants (Burgdorf et al., 2011). Whereas FLAT 50-kHz USV have been found to be more prominent in de-escalation of aggression as well as re-establishing social contact (Burke, Kisko, Pellis, et al., 2017; Wöhr, Houx, Schwarting, & Spruijt, 2008). In Study I, upon closer inspection it was revealed that, *Cacna1c* haploinsufficiency affected the 50-kHz USV profile by reducing FLAT and MIXED 50-kHz USV subtypes which have previously been associated with the synchronization of complex social interactions (Łopuch & Popik, 2011). Notably, specific play behaviors including pinning, wrestling, and chasing were differentially associated with certain 50-kHz USV subtypes and these further differed depending on genotype. The difference in subtypes was most prominently evident during chasing in *Cacna1c*+/- males, in which more TRILL 50-kHz USV were produced, but also to a lesser extent in wrestling in which high rates of both TRILL and FLAT 50-kHz USV were produced. In contrast, this subtype pattern was not observed in *Cacna1c*$^{+/+}$ rats, but rather MIXED 50-kHz USV were most prominent during chasing and wrestling. Pinning behaviors were associated with high rates of FLAT 50-kHz USV for both male *Canca1c*$^{+/-}$ and *Cacna1c*$^{+/+}$ littermates. In studies of pharmacological manipulations, TRILL 50-kHz USV are one of the most frequently produced 50-kHz USV after the administration of amphetamine and are thus, thought to be highly associated with the expression of a positive affective state (Rippberger et al., 2015). Additionally, Burgdorf and Panksepp (2006) suggest that specifically, the TRILL 50-kHz USV subtype is homologous to human laughter. Interestingly, during rough-and-tumble play in children, especially during chasing sustained bouts of laughter are evident (Smith & Lewis, 1985). In Study I, *Cacna1c*$^{+/-}$ males frequently produced TRILL calls during wrestling and especially chasing, suggesting that these behaviors may be particularly enjoyable for them, whereas this pattern was not as evident in *Cacna1c*$^{+/+}$ males. However, if it stands that MIXED and FLAT 50-kHz USV subtypes are more tightly linked to complex behaviors, as seen in male *Cacna1c* wildtype littermates in Study I, a substantially more detailed behavioral analysis of the specific playful

interactions may be necessary to see where the differences really lie. That is not to say that the current play behavior analysis in Study I and II was not detailed, in fact in comparison to the majority of studies investigating playful interactions where only pinning and overall play frequency is typically assessed (i.e., (Burgdorf, Moskal, Brudzynski, & Panksepp, 2013; Webber et al., 2012), the analysis of *Cacna1c* rough-and-tumble play in Study I and II is much more comprehensive. It is likely, that the specific structures of pinning and wrestling in *Cacna1c*$^{+/-}$ males may differ in comparison to wildtype controls. Using even more fine-tuned and precise analysis would allow a deeper look into the more specific behavioral alterations that may not be apparent using typical behavioral analysis. Indeed, some specific brain manipulations done by lesion and pharmacological studies have shown that specific differences in playful components can be altered (Pellis & Pellis, 2009). For example, when rats with no cortex reach peak play stages they still engage in rough-and-tumble play at rates similar to controls, however, the decorticate rats adopt a partial rotation defense tactic (i.e., the defender rotates the head, neck and shoulders, thereby withdrawing its nape from the attacker's snout but not becoming fully supine) as their primary preference and with the onset of puberty they continue to maintain the partial rotation preference, whereas, control rats during peak play stages adopt a complete rotation, where they lie fully supine on their back with all four paws in the air, and then at puberty switch to the partial rotation tactic (Pellis et al., 1992). Thus, if specific 50-kHz USV subtypes are more tightly linked to complex behavioral patterns in rough-and-tumble play, it is possible that this could be teased apart and become apparent in a more detailed behavioral analysis.

Additionally, FLAT 50-kHz USV are thought to be involved in (re)-establishing social contact, for example when conspecifics have been separated from their cagemates (Wöhr et al., 2008) and, moreover, have been linked to de-escalating aggression in adults (Burke, Kisko, Pellis, et al., 2017). Therefore, it is also likely that the FLAT USV is used when engaged in complex social interactions to maintain a playful atmosphere, however, escalation to aggressive attacks are very rarely observed in juvenile rough-and-tumble play (Pellis & Pellis, 2009) and this is likely not the role for this subtype in juvenile playful interactions, although to what specific role it is involved during juvenile play we are unable to say. Remarkably, to my knowledge this is the first study that is able to provide a direct comparison for specific play

behaviors in relationship to 50-kHz USV subtypes. Previous studies have investigated 50-kHz USV as specific play signals occurring prior to, or immediately following, playful interactions (B. T. Himmler et al., 2014) as well as, during the anticipatory phase of juvenile play in which only one rat is present in the play arena (Burke, Kisko, Swiftwolfe, et al., 2017), however, neither of these studies report the occurrence of specific subtypes during and within the actual playful behaviors. These previous studies indicate, similarly to Study I, that indeed 50-kHz USV emission and potentially the 50-kHz USV subtype profiles, may have a more important role than simply expressing the affective state as was hypothesized for example, by Knutson et al (1998). In contrast, however, Study I takes a more direct look at specific components of play and the relationship of 50-kHz USV emission, rather than assessing the USV as specifically play signals occurring to indicate an imminent attack (B. T. Himmler et al., 2014). Notably, the TRILL 50-kHz USV subtype appears to be the most common USV categorized during juvenile rough-and-tumble play with approximately 77% being TRILL subtypes and roughly 18% accounting for FLAT and MIXED 50-kHz subtypes (Himmler et al, 2014). The 50-kHz subtype profile for rough-and-tumble play in Study I does, however, appear to be consistent to the established findings, in that *Cacna1c* the wildtype 50-kHz USV subtype profile consists mainly of TRILL, FLAT and MIXED 50-kHz subtypes. Although, in Study I, the FLAT and MIXED 50-kHz USV do seem to have a larger proportion than what is reported in (Himmler et al, 2014), this may be due to classification and analysis differences. Additionally, strain differences may account for discrepancies in the 50-kHz USV subtypes in comparison to Study I, as Himmler et al (2014) used Long Evans rats. Evidence for strain differences in rough-and-tumble behavior suggests that this could possibly be something of importance (S. M. Himmler, Modlinska, Stryjek, et al., 2014). Study I, therefore, appears to support previously observed 50-kHz USV subtype profiles associated with juvenile rough-and-tumble play, however, further detailed assessments are warranted to draw any conclusive links.

In contrast to *Cacna1c*$^{+/-}$ males, female *Cacna1c*$^{+/-}$ juveniles do show behavioral alterations in rough-and-tumble play, specifically there was a significant increase in playful interactions, specifically pinning behavior, in comparison to the female wildtype siblings. This is in slight contrast to the original hypothesis expecting deficits rather than enhanced play behaviors but does support an expected sex difference in rough-and-tumble play, albeit in the opposite direction to what is typically observed (Pellis, 2002), suggesting that female

Cacna1c$^{+/-}$ rats exhibit more male-typical play patterns. Interestingly, however, *Cacna1c*$^{+/-}$ females had 50-kHz USV emission levels comparable to the wildtype controls. This indicates that unlike the male *Cacna1c*$^{+/-}$ rats, female *Cacna1c*$^{+/-}$ do not appear to display decrease in 50-kHz USV emission. This result is somewhat similar to findings in rats selectively bred for high and low levels of anxiety (Lukas & Wöhr, 2015). Female rats with lower levels of anxiety display much higher frequencies of playful interactions than high anxiety or control females, however 50-kHz USV emission does not differ between the low anxiety and control females (Lukas & Wöhr, 2015). Nevertheless, there are reports that female *Cacna1c* heterozygous mice have increased anxiety compared to controls (Dao et al., 2010), which would suggest similarly in our female heterozygous *Cacna1c* rats we should see a decrease rather than an increase in playful interactions, yet, this was not the case. Notably, in Dao et al (2010) increased anxiety was observed only in adult mice, as juveniles were not assessed. Therefore, this suggests that the increase in play behavior observed in *Cacna1c*$^{+/-}$ females is likely not driven by differences in anxiety levels or as result of increased 50-kHz USV emissions, when compared to wildtype littermate controls. In *Cacna1c* females, however, a detailed spectrographic and subtype analysis was not performed. Thus, it is possible that genotype differences may still exist in the subtype profiles and spectrographic properties, which could indicate impairments in incentive salience or reward. This could then possibly be observed through fewer TRILL 50-kHz USV which in turn may drive the increase in pinning behavior in an attempt achieve a higher reward level similar to wildtype *Cacna1c* females. As previously mentioned, TRILL 50-kHz USV are thought to be a reflection of the affective state, similar to laughter in humans (Burgdorf & Panksepp, 2006).

Typically, in many species the frequency of rough and tumble play is higher in males than in females (Auger & Olesen, 2009; Palagi & Paoli, 2007; Pellis, 2002; Pellis & Pellis, 1997; Takahashi et al., 1983; Thor & Holloway, 1986; Watson & Croft, 1993). Several brain regions responsible for expression of rough-and-tumble play, including cortical, limbic, hypothalamic, thalamic and sensory areas (Daenen et al., 2002; Vanderschuren et al., 2016) are responsive to sex steroid hormones (Shughrue et al, 1997; Simerly et al, 1990). Meaney & McEwen (1986) demonstrated that the amygdala was one such region that is important for sex differences in play because when they implanted testosterone capsules into the neonatal female rat amygdala their play behavior became masculinized. Furthermore, it was shown that dopaminergic

activation of estrogen receptors within the central amygdala and bed nucleus of the stria terminalis can masculinize social play in females (Olesen, Jessen, Auger, & Auger, 2005). More recently, however, a study by Argue & McCarthy (2015) investigated specific sex differences between male and female Sprague-Dawley rats. This is of note because our *Cacna1c* rat model is designed on a Sprague Dawley background and therefore the wildtype *Cacna1c* rats should be comparable to those used by Argue & McCarthy (2015). In parallel with our own play study pinning, chasing, as well as pouncing and boxing, of which we refer to as wrestling, were assessed in juvenile male and female rats between four and five weeks of age. Results indicate that in same-sex pairs males had a higher frequency of playful interactions than females and likewise, male pairs had higher rates of pinning, pouncing, and boxing in comparison to females although, the frequency of chasing between sexes did not differ (Argue & McCarthy, 2015). Interestingly, the frequency of pinning in male-female pairs in comparison to same sex pairs was significantly increased although not as high as male-male pairs which were still significantly higher in frequency and the same was true for pouncing and boxing rates as well as the overall frequency of playful interactions (Argue & McCarthy, 2015). Additionally, frequencies of chasing, in male-female pairs drastically increased in comparison to frequencies of same-sex pairs (Argue & McCarthy, 2015). Taken together these results suggest that the increased frequencies of playful interactions in our *Cacna1c*$^{+/-}$ females, and particularly the increase in pinning, could suggest that they are adopting a more masculinized play structure in comparison to wildtype control females. Notably, however, in Study II, both *Cacna1c*$^{+/-}$ and *Cacna1c*$^{+/+}$ males had lower rates of playful interactions, including pinning, when compared *Cacna1c*$^{+/-}$ females whereas, the wildtype *Cacna1c* females had rates that were roughly parallel to the males. This could therefore suggest that not only are the *Cacna1c*$^{+/-}$ females expressing a more masculinized rough-and-tumble play repertoire but potentially this may influence the wildtype *Cacna1c* females by increasing their overall play frequency as well. Indeed, Thor and Holloway (1986) found that the sex of cagemates can affect social behavior in males, although the same was not the case for females, nevertheless learning can reduce sex differences in play and the repeated play solicitation from males can increase females' play behaviors (Argue & McCarthy, 2015). Thus, if our *Cacna1c*$^{+/-}$ females are more masculinized, then repeated play solicitation with wild-type female cagemates could influence their subsequent levels of playful motivation. One possible way to investigate this could be to

group house females with same genotype cagemates and compare playful interactions. The source of more masculinized female play in *Cacna1c*$^{+/-}$ rats could, potentially, be due to impairments during the developmental trajectory of the amygdala as a consequence of decreased $Ca_v1.2$ expression levels. Several studies have shown that rough-and-tumble play can be altered by impairments in the amygdala during development (Kurian et al, 2008; Jessen et al, 2016; Taylor et al, 2012) resulting in sex differences. As we know from human *CACNA1C* studies, amygdala alterations do occur in healthy rs1006737 risk allele carriers (Lancaster, Foley, Tansey, Linden, & Caseras, 2016; Sumner, Sheridan, Drury, et al., 2015; Tesli et al., 2013). Another mechanism for the masculinized *Cacna1c*$^{+/-}$ female play could be that the *Cacna1c*$^{+/-}$ females have an altered testosterone level, which again could have resulted from impairments or alterations during development of androgen regulation in specific brain regions. One future possibility could be to assess androgen levels at different stages of development in *Cacna1c* female rats. There is some suggestion that the masculinized behaviors of *Cacna1c*$^{+/-}$ females tend to persist and remain into adulthood, with the heterozygous *Cacna1c* females displaying a higher rate of dominance than female wildtype *Cacna1c* controls in a tube test (unpublished, PhD thesis work - Tobias Redecker) however, this finding and interpretation needs to be further explored through subsequent testing and analysis.

Our findings during the pro-social 50-kHz playback paradigm are mostly in line with the original hypothesis that *Cacna1c* haploinsufficiency leads to a blunted or reduced response for 50-kHz USV playback, though there was a minor difference between the sexes which was not expected. During pro-social 50-kHz USV playback *Cacna1c*$^{+/-}$ males were observed to approach the ultrasonic speaker emitting 50-kHz USV, however, the preference for the proximal arms nearest to the speaker during playback was higher in the wildtype controls in comparison. In contrast females of both genotypes responded with increased preference for the proximal arms at comparable rates. Additionally, in the minutes following 50-kHz USV playback wildtype *Cacna1c* males and females continued to display a preference for the proximal arms indicating a motivation to continue searching for the source of the USV. Whereas, *Cacna1c*$^{+/-}$ males and *Cacna1c*$^{+/-}$ females showed no preference for the proximal or distal arms in the minutes following playback, suggesting a possible lack of social incentive salience.

There are relatively few studies investigating social behavior in *Cacna1c* mouse models and of those that do, it is adult social behavior which is examined, typically using a three-chambered social approach and social memory test. Nonetheless, there have been some relevant findings on the social behavior of *Cacna1c* mice. In mouse models in which there is a gain of function in $Ca_v1.2$ channels, namely the TS2-neo mice, there are no impairments in sociability when tested in the standard three-chambered social approach assay (Bader et al., 2011; Kabitzke et al., 2017). However, in a prolonged three-chamber test Bader et al (2011) showed that the TS2-neo mice lose the preference for the novel mouse after the first hour and instead show the opposite behavior over the remaining duration. This suggests that there may be a more distinct deficit in the TS2-neo mice in regards to social preference that could indicate an impairment in incentive-motivation for social interactions that wasn't evident in the standard assay. This finding is somewhat in parallel with our findings in *Cacna1c*$^{+/-}$ rats during 50-kHz USV playback. In Study I and II, social approach was evident in response to 50-kHz USV playback, however, in the minutes following *Cacna1c*$^{+/-}$ males and females showed no preference for the proximal or distal arms possibly indicating a diminished incentive-motivational state. Though, in a test for social memory both wildtype and TS2-neo mice performed equally as well showing a higher preference, or dishabituation, for the novel mouse (Bader et al, 2011). This finding was later substantiated by Kabizke et al (2017). Sociability deficits were also, found in a mouse models in which there is a loss of $Ca_v1.2$ function. In conditional forebrain *Cacna1c* knockout (fbKO) mice deficits in sociability were seen during a standard three-chambered social assay. *Cacna1c* fbKO mice have no preference for either the stranger mouse or the novel object and spend equal amounts of time with both, whereas the wildtype controls spent more time with the stranger mouse (Kabir et al., 2017). Additionally, focal ablation of *Cacna1c* within the PFC also resulted in decreased sociability suggesting that particularly within this region of the brain expression of $Ca_v1.2$ is associated with social behavior (Kabitzke et al., 2017). Taken together this indicates that when there is a disruption in $Ca_v1.2$ channels resulting in altered expression levels, social incentive-motivation may be impaired which could potentially lead to impairments in recognizing emotionally valenced stimuli, such as 50-kHz USV in Study I and II. In human studies, the PFC, as well as amygdala and hippocampal, alterations have been shown to lead to impairments in emotional learning tasks involving emotionally valanced social stimuli in the form of human faces

(Bolton, Heaney, Sabbagh, et al., 2012). Interestingly, it has been found that volume (Dietsche et al., 2014; Perrier et al., 2011; Wang et al., 2011) and function (Bigos et al., 2010; Dima et al., 2013; Jogia et al., 2011; Radua et al., 2013; Tesli et al., 2013) of structures, such as the PFC, are altered in *CACNA1C* rs1006737 risk allele carriers and this in turn appears, to alter their response to both positive and negative emotionally valanced social stimuli (Dima et al., 2013; Pasparakis et al., 2015; Roussos et al., 2011).

To date, in *Cacna1c* animal models there have been no positive emotional learning tests done but some researchers have explored the effects of negative emotional learning by means of fear conditioning. Typically, fear learning paradigms are used to measure emotional learning in animals (Phelps & LeDoux, 2005). However, some studies have found no effects on fear learning using contextually-cue associated fear memories (Langwieser et al., 2010; McKinney et al., 2008) and suggested that this may be due to compensatory effects within the amygdala in brain specific *Cacna1c* knockout mice (Langwieser et al., 2010). Kabir et al (2016) has suggested that the use of developmental knockout mouse models such as those used by Langwieser et al (2010) and McKinney et al (2008) may result in compensatory adaptations that override the influence of $Ca_v1.2$ LTCCs in fear memory paradigms and, thus, these specific mouse models may not be the optimal candidates to study the role of $Ca_v1.2$ channels in the processing of fear memories (Kabir et al., 2016). Evidence supporting this theory is shown in studies using pharmacological LTCC blockers. For example, acute inhibition of LTCCs at specific sites of the brain is sufficient to induce deficits in recall of cue-associated fear memories (Bauer et al., 2002; Langwieser et al., 2010) as well as, with repeated inhibition leading to impairments in cue fear extinction (Davis & Bauer, 2012). This points to a role for LTCCs in mediating the processing of fear memories (Davis & Bauer, 2012), however, notably, there is no differentiation between $Ca_v1.2$ and $Ca_v1.3$ channels when using LTCC blockers. One method suggested by Kabir et al (2016) to further explore the role of $Ca_v1.2$ channels specifically, is to use viral vectors such as those used by Lee et al (2012). When employing this method in *Cacna1c* fbKO mice, Kabir et al (2017) established that during training *Cacna1c* fbKO mice and controls displayed no deficits in fear acquisition to cue or context, however, during testing *Cacna1c* fbKO mice showed higher freezing to the cue but not the context, which may be an indication of anxiety level and not emotional learning, *per se*. Also using the method established by Lee et al (2012), in one *Cacna1c* mouse model it has

been found that during observational fear learning, i.e., one mouse observes another receive repeated foot shocks, conditional knockout of $Ca_v1.2$ in the ACC resulted in fear learning impairments (Jeon et al., 2010). While information on *Cacna1c* and fear learning is limited, Jeon et al (2010) provides evidence for a social aspect in learning. Notably, the ACC $Ca_v1.2$ knockout mice showed anxiety and innate fear levels comparable to controls during open field, EPM, light-dark box, novel object recognition and predator exposure, suggesting that neural mechanisms underlying observational social fear may be different from those of innate fear and anxiety (Jeon et al., 2010). Moreover, during classical fear conditioning ACC $Ca_v1.2$ mice had a normal fear response further suggesting that investigation into the social fear learning mechanisms is necessary (Jeon et al., 2010). Thus, through negative emotional learning it seems like there is conflicting evidence for the role of *Cacna1c* however, a social influence does appear to affect $Ca_v1.2$ brain specific knockout *Cacna1c* models. During fear conditioning the rate of freezing is what is commonly measured and the freezing rate is highly correlated with the emission of 22-kHz USV (Wöhr & Schwarting, 2008b). This response can be blocked, however, by inactivation of the amygdala (Brudzynski, 2013) supporting a more specific role for the amygdala in emotional-context learning. Therefore, if *Cacna1c*$^{+/-}$ male rats have alterations in their amygdala during development which subsequently impairs their ability to associate emotionally valanced stimuli in specific contexts; as observed in the reduced preference for proximal arms during 50-kHz USV playback, one possibility to further investigate this could be through an observational social learning task. Methods to investigate positive emotional learning however, are needed to further fully explore this hypothesis.

Cacna1c Haploinsufficiency Strengthens Cognitive Abilities and Creates Sex-Specific Effects in Learning

Although the main components of neuropsychiatric disorders generally discussed are alterations in mood and emotion, deficits in cognitive functioning can also negatively impact the lifestyle of the individual (Millan, Agid, Brüne, et al., 2012). Several human studies have indeed shown an association between *CACNA1C* rs1006737 and cognitive functions (Dietsche et al., 2014; Kabir et al., 2017; Krug et al., 2010; Thimm et al., 2010; Zanos et al., 2015; Q. Zhang et al., 2011). Although, in contrast some studies have reported no significant impact in cognition in healthy risk allele carriers (Rolstad, Sellgren Majkowitz, Joas, et al., 2016;

Roussos et al., 2011; Soeiro-de-Souza et al., 2013). *Cacna1c* mouse models have reflected human findings and similarly provided indecisive results. However, one important paradigm focusing on particular forms of cognitive function, namely reversal learning and cognitive flexibility are absent from any relevant *Cacna1c* animal models. It may also be inferred that with the rich behavioral repertoire encompassing enhanced social behavior, increased sensitivity to rewards and more efficient learning strategies, rats represent a more ideal candidate to study and validate cross-species validation of *Cacna1c* gene deletion effects (Ellenbroek & Youn, 2016). Thus, it was hypothesized that through rats, further cognitive alterations resulting from haploinsufficiency could become apparent. Our results, however, show that neither *Cacna1c*$^{+/-}$ nor *Cacna1c*$^{+/+}$ rats exhibit cognitive impairments and this was largely true for both males and females.

In general, *Cacna1c* rats appear to show normal, and in some cases above normal, spatial learning over a seven-day period. However, during reversal learning male *Cacna1c*$^{+/-}$ rats displayed reduced cognitive flexibility. This finding is in contrast to the original hypothesis expecting results similar to deficits seen in *Cacna1c* mouse models (i.e., (Dedic et al., 2017; Moosmang et al., 2005)). Importantly, in comparison with other *Cacna1c* mouse studies the distinction between spatial reference memory and spatial working memory needs to be mentioned, as the radial arm maze task used in Study III assesses both. The T- and Y-mazes, focus on spatial working memory whereas the MWM primarily targets reference memory (Morellini, 2013). These paradigms also exert their effects through involvement of the PFC, hippocampus and dorsal striatum (Morellini, 2013). Specifically, the PFC has been shown through other animal studies to be a central anatomical structure mediating high-order cognitive functioning by means of top-down executive control on subcortical structures such as the hippocampus, striatum, thalamus and amygdala (Miller, 2000; Riga, Matos, Glas, et al., 2014). As mentioned previously, functional imaging studies in humans rs1006737 risk allele carriers have identified increased activity in PFC during a working memory task suggesting a decreased efficiency in PFC (Bigos et al., 2010) On the other hand, however, Erk et al (2014) and Paulus et al (2014) both found decreased PFC activation and altered connectivity between PFC and hippocampus. Nevertheless, increased or decreased functioning both point to potential alterations in PFC functioning and connectivity which subsequently can impact accompanying structures. In a study by White et al (2008) the authors used a conditional

forebrain knockout *Cacna1c* mouse model and assessed spatial memory discrimination in the MWM. Similar to our findings, White et al (2008) found no impairment in spatial learning over a 14-day testing period. During probe trials, within testing days, no differences between genotypes were detected by the authors, suggesting that all animals were able to learn appropriately and retain that information, as was the case in our study, 30 days after testing though, White et al (2008) found the *Cacna1c* forebrain knockouts were no longer able to remember the location of the platform. Therefore, cognition and memory may appear to be intact, but, the consolidation of remote spatial learning memories are diminished. This may suggest that impairments resulting from a *Cacna1c* decrease may not be immediately apparent and may, in fact, be task-time dependent. In our Study III, we did not test past the 14-day learning and relearning period and therefore these impairments could possibly exist in the *Cacna1c*$^{+/-}$ rat as well, further long-term investigation is warranted. Temme et al (2016) provides further evidence for an intact spatial memory in conditional *Cacna1c* knockout mice, however, when the task was made more difficult the impairments became much more evident in the knockouts when compared to wildtype mice. Additionally, no differences have been detected using a T-maze paradigm in heterozygous *Cacna1c* mice (Kabitzke et al., 2017).

Moosmang et al (2005) on the other hand, did find spatial memory impairments during the MWM task using a *Cacna1c* mouse model. This task however is hippocampus dependent and the authors specifically used complete inactivation of the *Cacna1c* gene in the hippocampus and neocortex. Our own *Cacna1c* rat model is a global, i.e., whole body and brain, elimination for one copy of the *Cacna1c* gene and thus, it may be that impairments are only evident when a complete knockout is done in specific brain regions that are required for spatial tasks and cognitive flexibility. An argument, nonetheless, can also be made from the studies done using *Canca1c* mouse models, in regards to the type of learning which may be impaired by *Cacna1c* gene manipulations. For an additional example, mice lacking *Cacna1c* in the hippocampus and cortex display no impairments during a task of contextual fear learning or extinction (McKinney et al., 2008) but do during the MWM (Moosmang et al., 2005), suggesting that alterations in behavioral responses during learning and memory tasks may be dependent no only on the availability of $Ca_v1.2$ expression but appear to also rely on other factors and mechanisms involved in the task. This becomes more evident in an observational fear learning task, in which ACC deletion of $Ca_v1.2$ impaired the social observation fear learning but not

classical fear learning (Jeon et al., 2010). It is likely, as well, that our haploinsufficient *Cacna1c* rat model is not specific enough to manifest impairments in cognition, as is the case in more brain specific knockouts. Indeed, it has even been suggested that deficiencies of *Cacna1c* could lead to protective rather than harmful effects (Dao et al., 2010; Zanos et al., 2015).

Looking more specifically at the effects of haploinsufficiency in *Cacna1c* rats, we did observe some minor sex specific effects during the spatial learning and relearning tasks. Heterozygous males, for example, appear to have a more robust memory trace for the locations of rewards, which is in direct contrast to the Moosmang et al (2005) and White et al (2008) findings and further was not seen in heterozygous *Cacna1c* females. However, this effect was only short term and did not persist for the entire duration of spatial relearning. This suggests that perhaps the cognitive flexibility is slightly altered by a decrease in *Cacna1c* in rats, but this is not enough to result in persistent impairment effects. Female heterozygous *Cacna1c* rats displayed an initial hypo-activity which resulted in a slightly better performance than the wildtype *Cacna1c* females, however, the hypoactive behavior did not persist during testing and did not impair accuracy in relation to wildtype *Cacna1c* females. In *Cacna1c* mouse models, the information about sex-specific effects are sparse, however, some researchers have found an increased anxiety phenotype in females (Dao et al, 2010), which could suggest an initial hesitation in locomotor behavior observed in our *Cacna1c* heterozygous females. Although relatively minor, the sex-specific effects seen in adult *Cacna1c* rats could be a result of effects of decreases in $Ca_v1.2$ during development when the brain begins to differentiate male and female species-specific behaviors (Lenz, Nugent, & McCarthy, 2012). Although, a hypoactive female is in contrast to the effects observed in post-weaning adolescents in Study I and II. This suggests that hormonal and age-related effects may also influence behavioral shifts and outcomes in *Cacna1c* heterozygous rats. Zanos et al (2015) investigated aging effects in a similarly heterozygous *Cacna1c* mouse model and found a genotype difference driven by older groups of mice, suggesting that more longitudinal testing could be done in *Cacna1c* haploinsufficient rats to see if there is a similar effect to that of constitutive *Cacna1c* mice. Alternatively, there may be compensatory processes at work in the heterozygous rats which could rescue or even mask impairments (Salama & London, 2007).

Cacna1c Haploinsufficient Rat Model: A Translational Perspective

Social behavior and communication deficits are core features for both affective and neurodevelopmental disorders and in humans any impairments in these domains could easily result in a reduced quality of life. Additionally, cognitive impairments seen in these disorders may influence and impair social functioning and vice versa, suggesting that it is a catch-22 scenario. As a result, either cognitive or social impairments may severely impact the overall life quality for patients diagnosed with severe neuropsychiatric disorders. In that regard, behavioral phenotyping of rat models for neuropsychiatric disorders, characterized by social and cognitive impairments can help provide insight into the basis of the disorder-relevant impairments. Through two reviews and three empirical studies, this dissertation aimed to provide a connection between two species, with detailed attention to the specific relationship with human symptoms. Based on our findings from the empirical studies, certain analogous impairments were observed in terms of social interaction, communication, learning and memory, as well as sex-specific differentiation.

In humans, GWAS have shown that healthy rs1006737 risk allele carriers have alterations in social behavior (Dima et al., 2013; Pasparakis et al., 2015; Roussos et al., 2011), verbal communication skills (Erk, Meyer-Lindenberg, Linden, et al., 2014; Erk et al., 2010; Krug et al., 2010; Roussos et al., 2011) and cognitive functioning (Hori et al., 2013; Soeiro-de-Souza et al., 2013), specifically in attention (Thimm et al., 2010), working memory (Q. Zhang et al., 2011), and recognition memory (Dietsche et al., 2014). This strongly suggests that the *CACNA1C* gene may have an influential role in the phenotypic expressions for social functioning and cognition in humans. An important study by Dao et al (2010) further showed a supportive gene by sex interaction that was paralleled in human and animal data. Importantly, Dao et al (2010) used a *Cacna1c* conditional heterozygous mouse model that was similar to our own rat model, in that there was a global decrease of $Ca_v1.2$ expression. An important finding from this study was the increased anxiety seen in female mice and human *CACNA1C* risk allele carries, although notably their findings were in different SNPs, namely rs2370419 and rs2470411 (Dao et al., 2010). To my knowledge, this is the only study with parallel findings in human and mouse *Cacna1c* data that specifically investigated both sexes. Moreover, some of the largest effects seen in our own behavioral data was observed in our

female heterozygous *Cacna1c* rats suggesting a strong likelihood that alterations in the $Ca_v1.2$ channel may be more evident in the behavior of females. In affective disorders, such as MDD the prevalence rates are approximately twice as high in women than men (Kuehner, 2003) and in BPD women tend to experience more depressive episodes and are at an increased risk for a form of rapid cycling (Kendler, Gatz, Gardner, & Pedersen, 2006).

While in the study by Dao et al (2010) there was strong anxiety behavior in *Cacna1c* heterozygous female mice, we found the opposite and in fact, our *Cacna1c* heterozygous female rats were more socially motivated, as demonstrated by the increased playful interactions and pinning behavior. This may be because the females used by Dao et al (2010) were adults and thus, past the puberty stage. In humans the strong prevalence rates of MDD in women are much more evident past puberty (Kuehner, 2003). Through other behavioral data collected as part of a larger *Cacna1c* rat phenotyping project, we have observed higher anxiety in the *Cacna1c*$^{+/-}$ female rats. Conversely, in *Cacna1c*$^{+/-}$ males, specific impairments were observed in emission and response to social communication, by means of 50-kHz USV. Strong social communication impairments are characteristic of neurodevelopmental disorders, such as ASD. SHANK mouse models have become an ideal transgenic model with promising insight into mechanisms, causes, treatments and the overall etiology of ASD (Sungur et al., 2017; Yoo et al., 2014). Recently, SHANK scaffolding proteins strongly associated with ASD (Monteiro & Feng, 2017) have been implicated in the regulation of LTCCs, including $Ca_v1.2$ channels (Pym et al., 2017). Therefore, mutations in the SHANK gene family could lead to malfunctions or irregularities in $Ca_v1.2$, potentially contributing to ASD-related phenotypes, such as communication impairments. Most research with SHANK mutations, however, use mouse models, and in terms of juvenile social behaviors, mice are not the ideal candidate (Ellenbroek & Youn, 2016; Homberg et al., 2017). A SHANK3 rat model has been developed in which some diminished impairments in response to playback of 50-kHz USV were observed, (Berg, Copping, Rivera, et al., 2018) however, no information is available on juvenile social play behavior or concomitant 50-kHz USV emission. Therefore, taken together, it seems apparent that our haploinsufficient *Cacna1c* rat model is an ideal candidate to study the relevant translational effects of social impairments seen in affective and neurodevelopmental disorders.

Future Perspectives

Broadening known information from previous reports, the findings from two reviews and three empirical studies presented here, support persuasive evidence for disorder-relevant features in a haploinsufficient *Cacna1c* rat model for affective and neurodevelopmental disorders. Behavioral phenotyping from an early developmental stage enables an in-depth source of material for the characterization of behavioral abnormalities underlying affective and neurodevelopmental deficits and can further serve as a valid tool to develop potential therapeutic approaches targeting more specific impairments.

By determining more specifically the underlying mechanisms causing the impairments, as well as conducting full phenotyping assays, methods can be developed to help rescue the deficits and contribute to an overall increase in the quality of life and decrease prevalence of such detrimental disorders world-wide. For example, through detailed phenotyping of rats bred specifically for high and low rates of 50-kHz USV emission, Burgdorf et al (2017) was able to create a targeted treatment plan to help combat depression in humans. This was done by using a positive emotional learning paradigm and identifying NMDAR-dependent synaptic plasticity in the mPFC as the critical site mediating positive emotional learning. Due to the strong association of dopamine in the sender and receiver of 50-kHz USV and the report that dopamine signaling is impaired in *Cacna1c* mouse models (Kabir et al., 2016; Terrillion, Dao, et al., 2017), further investigation seems necessary to tease apart the alterations in dopamine during positive rewarding social interactions, in which typically enhanced release of dopamine is observed (Willuhn et al., 2014). Additionally, a lack of social approach response to 50-kHz USV shown in Study I and II in male *Cacna1c*$^{+/-}$ and *Cacna1c*$^{+/-}$ females, could reflect a blunted emotional or apathetic response similarly seen in humans diagnosed with affective or neurodevelopmental disorders (American Psychiatric Association, 2013). The distinction between behavior and USV results suggests that further investigation into the mechanisms regulating playful interactions in male and female *Cacna1c* rats is reasonable. One recommendation could be to investigate the levels of dopamine by means of voltammetry, following playful interactions and 50-kHz USV playback. A strong likelihood worth mentioning as well is that for *Cacna1c*$^{+/-}$ males 50-kHz USV may not have as much emotional value in relation to the behavioral outcome. Conceivably, during development the diminished

50-kHz USV emission resulting from potentially altered dopamine signaling, decreases the emotional salience gained from contextual learning. Some evidence to support this was shown in contextual fear conditioning in *Cacna1c* fbKO mice, which do not show contextual fear responses (Kabir et al, 2017). In order to further explore and investigate the emotional salience from contextual learning surgical devocalization of the play partners could be performed post-weaning and then the subsequent effects on the playful interactions assessed. If *Cacna1c*$^{+/-}$ males experience a blunted pleasure response or have reduced emotional value for 50-kHz USV, then a much less drastic decrease in the rough-and-tumble play might result in comparison to wildtype controls. Additionally, pairing a devocalized *Cacna1c*$^{+/-}$ with an intact *Cacna1c*$^{+/-}$ or wildtype control could further investigate the level at which 50-kHz USV emission begins to contribute to the overall playful motivation, reflected in the frequency of playful interactions. Playback of 50-kHz USV might also be a possible rescue or assessment measure for emotional value. However, it is important to point out that there is likely an optimal level of dopamine required in order for rough-and-tumble play to occur in rats (Trezza, Baarendse, & Vanderschuren, 2010) and likewise, it then seems reasonable that there would be a maximum level of dopamine release which would create a ceiling effect, resulting in no changes to playful frequencies (Vanderschuren, Trezza, Griffioen-Roose, et al., 2008). Thus, playful interactions themselves may stimulate the maximum availability for dopamine release in *Cacana1c*$^{+/-}$ rats in which case 50-kHz USV emission would not add any further rewarding neuronal stimulation. Again, this could be further investigated through either *in vivo* or *in vitro* neurotransmitter levels. Moreover, further investigation into more specific side-effects of the devocalization procedure and more specifically the effects on the development of the dopaminergic system within the brain would need to be done to rule out interaction effects or confounds of the surgery.

In females a more in-depth assessment of androgens could also be investigated, with specific focus on testosterone levels. Fully exploring a variety of positive social interactions, such as social recognition memory, in which 50-kHz USV are emitted, would also be helpful to determine specific components of the social interaction that rats may, or may not, find to be rewarding. Likewise, interconnections between and within structures such as the PFC-amygdala-hippocampus circuit are required to establish suitable social functioning and therefore, subsequent cognitive abilities such as, planning or problem-solving strategies, the

emotional processing of information or social cognition are likely needed to cope satisfactorily with different psychosocial situations or events. Thus, a more in-depth analysis of specific brain regions and their connectivity to each other would be essential, especially, when using a more specific analysis of each individual during a social interaction. This might reveal specific individual behavioral alterations, masked by assessing the behavior as a pair, especially in terms of social play. Several playful interactions are brain site-specific and damage to one of these areas can lead to impairments in the regulation of social play (Pellis & Pellis, 2009).

Within the brain, $Ca_v1.2$ and $Ca_v1.3$ channels both exist, and thus it may be likely that specific impairments are more associated with certain channel alterations. Alternatively, the absence of impairment could indicate compensatory mechanisms. Restoring $Ca_v1.2$ in certain brain regions, creating a more specific brain site knockout or knockin using viral vectors such as those used in mice by Lee et al (2012) or Bader et al (2011), or investigating mRNA manipulations could help isolate regions of the brain in which differential $Ca_v1.2$ expression levels affect the social behavior and communication aspects of neuropsychiatric disorders and by means of rat models these early social impairments could be easily assessed by social play and 50-kHz USV playback methods.

Concluding Remarks

"Ja, Kalzium, das ist alles!"[*]

Otto Loewi -1959

Although spoken in terms of smooth muscle contraction, Otto Loewi had no idea how relevant to the field of science, and in this regard to behavioral neuroscience, this statement really was. As it turns out alterations in the *Cacna1c* gene as a result of decreased $Ca_v1.2$ expression, can lead to disorder-like phenotypes in social behavior and pro-social communication associated with affective and neurodevelopmental disorders. Thus, the relationship between behavior and genetics is essential to help understand the complex etiology and pathophysiology of severe neuropsychiatric disorders. With recent advancements in the field of behavioral neuroscience and genetics the creation of novel transgenic animal models helps bridge the gap a little more and brings us closer to finding effective treatments and therapies for those who suffer. By using a novel *Cacna1c* rat model this dissertation aimed to further explore the role of $Ca_v1.2$ expression levels in social behavior, ultrasonic communication and cognition, with importance to deficits observed in neuropsychiatric disorders. As a result of several important experiments there are some promising new leads to the underlying mechanisms opening the door for further research into treatment possibilities. *Cacna1c* haploinsufficiency in rats provides an encouraging new model for social communication and incentive salience deficits characterizing MDD, BPD, SCZ and ASD. Together, findings presented in this dissertation provide several in-depth behavioral paradigms and a promising new animal model to assess the development of social behavior and communication, as well as cognitive abilities, with robust relevance to neuropsychiatric disorders.

[*] Otto Loewi (1873-1961) – winner of 1936 Nobel Prize in Physiology or Medicine

Bibliography

Achterberg, E. J. M., van Kerkhof, L. W. M., Servadio, M., van Swieten, M. M. H., Houwing, D. J., Aalderink, M., … Vanderschuren, L. J. (2016). Contrasting Roles of Dopamine and Noradrenaline in the Motivational Properties of Social Play Behavior in Rats. *Neuropsychopharmacology*, *41*(3), 858–868. https://doi.org/10.1038/npp.2015.212

Ahrens, A. M., Nobile, C. W., Page, L. E., Maier, E. Y., Duvauchelle, C. L., & Schallert, T. (2013). Individual differences in the conditioned and unconditioned rat 50-kHz ultrasonic vocalizations elicited by repeated amphetamine exposure. *Psychopharmacology*, *229*(4), 687–700. https://doi.org/10.1007/s00213-013-3130-9

Alberini, C. M., Ghirardl, M., Metz, R., & Kandel, E. R. (1994). C/EBP is an immediate-early gene required for the consolidation of long-term facilitation in Aplysia. *Cell*, *76*(6), 1099–1114. https://doi.org/10.1016/0092-8674(94)90386-7

Allen Institute for Brain Science. (2004). Gene: *CACNA1C*. Retrieved from http://mouse.brain-map.org/gene/show/12073

American Psychiatric Association. (2013). *Diagnostic and statistical manual of mental disorders (DSM-5®)* (5th ed.). Washington, DC.

Anderson, S. M., Famous, K. R., Sadri-Vakili, G., Kumaresan, V., Schmidt, H. D., Bass, C. E., … Pierce, R. C. (2008). CaMKII: a biochemical bridge linking accumbens dopamine and glutamate systems in cocaine seeking. *Nature Neuroscience*, *11*(3), 344–353. https://doi.org/10.1038/nn2054

Argue, K. J., & McCarthy, M. M. (2015). Characterization of juvenile play in rats: importance of sex of self and sex of partner. *Biology of Sex Differences*, *6*(1), 16. https://doi.org/10.1186/s13293-015-0034-x

Arts, B., Simons, C. J. P., & Os, J. van. (2013). Evidence for the impact of the *CACNA1C* risk allele rs1006737 on 2-year cognitive functioning in bipolar disorder. *Psychiatric Genetics*, *23*(1), 41–42. https://doi.org/10.1097/YPG.0b013e328358641c

Assini, R., Sirotin, Y. B., & Laplagne, D. A. (2013). Rapid triggering of vocalizations following social interactions. *Current Biology*, *23*(22), R996–R997.

Auger, A. P., & Olesen, K. M. (2009). Brain Sex Differences and the Organisation of Juvenile Social Play Behaviour. *Journal of Neuroendocrinology*, *21*(6), 519–525. https://doi.org/10.1111/j.1365-2826.2009.01871.x

Austin, M. P., Mitchell, P., & Goodwin, G. M. (2001). Cognitive deficits in depression: possible implications for functional neuropathology. *The British Journal of Psychiatry : The Journal of Mental Science*, *178*, 200–206. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11230029

Backes, H., Dietsche, B., Nagels, A., Konrad, C., Witt, S. H., Rietschel, M., … Krug, A. (2014). Genetic variation in *CACNA1C* affects neural processing in major depression. *Journal of Psychiatric Research*, *53*(1), 38–46.

Bader, P. L., Faizi, M., Kim, L. H., Owen, S. F., Tadross, M. R., Alfa, R. W., … Shamloo, M. (2011). Mouse model of Timothy syndrome recapitulates triad of autistic traits. *Proceedings of the National Academy of Sciences*, *108*(37), 15432–15437.

Baenninger, L. P. (1966). The reliability of dominance orders in rats. *Animal Behaviour*, *14*(2–3), 367–371. https://doi.org/10.1016/S0003-3472(66)80099-4

Bailey, A., Le Couteur, A., Gottesman, I., Bolton, P., Simonoff, E., Yuzda, E., & Rutter, M. (1995). Autism as a strongly genetic disorder: evidence from a British twin study. *Psychological Medicine*, *25*(1), 63-67. https://doi.org/10.1017/S0033291700028099

Barch, D. M., & Ceaser, A. (2012). Cognition in schizophrenia: core psychological and neural mechanisms. *Trends in Cognitive Sciences*, *16*(1), 27–34.

Barfield, R. J., & Thomas, D. A. (1986). The Role of Ultrasonic Vocalizations in the Regulation of Reproduction in Rats. *Annals of the New York Academy of Sciences*, *474*(1), 33–43. https://doi.org/10.1111/j.1749-6632.1986.tb27996.x

Bari, A., Theobald, D. E., Caprioli, D., Mar, A. C., Aidoo-Micah, A., Dalley, J. W., & Robbins, T. W. (2010). Serotonin Modulates Sensitivity to Reward and Negative Feedback in a Probabilistic Reversal Learning Task in Rats. *Neuropsychopharmacology*, *35*(6), 1290–1301. https://doi.org/10.1038/npp.2009.233

Barker, D. J., Bercovicz, D., Servilio, L. C., Simmons, S. J., Ma, S., Root, D. H., … West, M. O. (2014). Rat ultrasonic vocalizations demonstrate that the motivation to contextually reinstate cocaine-seeking behavior does not necessarily involve a hedonic response. *Addiction Biology*, *19*(5), 781–790. https://doi.org/10.1111/adb.12044

Barker, D. J., Root, D. H., Ma, S., Jha, S., Megehee, L., Pawlak, A. P., & West, M. O. (2010). Dose-dependent differences in short ultrasonic vocalizations emitted by rats during cocaine self-administration. *Psychopharmacology*, *211*(4), 435–442.

Barker, D. J., Simmons, S. J., Servilio, L. C., Bercovicz, D., Ma, S., Root, D. H., … West, M. O. (2014). Ultrasonic vocalizations: evidence for an affective opponent process during cocaine self-administration. *Psychopharmacology*, *231*(5), 909–918.

Barnett, J. H., Sahakian, B. J., Werners, U., Hill, K. E., Brazil, R., Gallagher, O., … Jones, P. B. (2005). Visuospatial learning and executive function are independently impaired in first-episode psychosis. *Psychological Medicine*, *35*(7), 1031–1041.

Barnett, S. A., & Spencer, M. M. (1951). Feeding, Social Behaviour and Interspecific Competition in Wild Rats. *Behaviour*, *3*(3), 229–242.

Baron-Cohen, S., Ring, H. A., Bullmore, E. T., Wheelwright, S., Ashwin, C., & Williams, S. C. R. (2000). The amygdala theory of autism. *Neuroscience & Biobehavioral Reviews*, *24*(3), 355–364. https://doi.org/10.1016/S0149-7634(00)00011-7

Barrett, C. F., & Tsien, R. W. (2008). The Timothy syndrome mutation differentially affects voltage- and calcium-dependent inactivation of Ca(V)1.2 L-type calcium channels. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(6), 2157–2162. https://doi.org/10.1073/pnas.0710501105

Bauer, E. P., Schafe, G. E., & LeDoux, J. E. (2002). NMDA receptors and L-type voltage-gated calcium channels contribute to long-term potentiation and different components of fear memory formation in the lateral amygdala. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *22*(12), 5239–5249.

Bell, H. C., Pellis, S. M., & Kolb, B. (2010). Juvenile peer play experience and the development of the orbitofrontal and medial prefrontal cortices. *Behavioural Brain Research*, *207*(1), 7–13. https://doi.org/10.1016/j.bbr.2009.09.029

Bell, R., & Nitschke, W. (1974). Early experience, ultrasonic vocalizations, and maternal responsiveness in rats. *Developmental Psychobiology, 7*(3), 235–242.

Berg, E. L., Copping, N. A., Rivera, J. K., Pride, M. C., Careaga, M., Bauman, M. D., … Silverman, J. L. (2018). Developmental social communication deficits in the Shank3 rat model of phelan-mcdermid syndrome and autism spectrum disorder. *Autism Research, 11(4),* 587-601. https://doi.org/10.1002/aur.1925

Berger, S. M., & Bartsch, D. (2014). The role of L-type voltage-gated calcium channels Cav1.2 and Cav1.3 in normal and pathological brain function. *Cell and Tissue Research*, *357*(2), 463–476. https://doi.org/10.1007/s00441-014-1936-3

Bergner, C. L., Smolinsky, A. N., Hart, P. C., Dufour, B. D., Egan, R. J., LaPorte, J. L., & Kalueff, A. V. (2010). Mouse Models for Studying Depression-Like States and Antidepressant Drugs. In G. Proetzel & M. V Wiles (Eds.) Mouse Models for Drug Discovery. Methods in Molecular Biology (Methods and Protocols), vol 602 (pp. 267–282). Totowa, NJ: Humana Press. https://doi.org/10.1007/978-1-60761-058-8_16

Berridge, K. C. (2007). The debate over dopamine's role in reward: the case for incentive salience. *Psychopharmacology*, *191*(3), 391–431. https://doi.org/10.1007/s00213-006-0578-x

Bhat, S., Dao, D. T., Terrillion, C. E., Arad, M., Smith, R. J., Soldatov, N. M., & Gould, T. D. (2012). *CACNA1C* (Cav1.2) in the pathophysiology of psychiatric disease. *Progress in Neurobiology*, *99*, 1–14. https://doi.org/10.1016/j.pneurobio.2012.06.001

Bigos, K. L., Mattay, V. S., Callicott, J. H., Straub, R. E., Vakkalanka, R., Kolachana, B., … Weinberger, D. R. (2010). Genetic Variation in *CACNA1C* Affects Brain Circuitries Related to Mental Illness. *Archives of General Psychiatry*, *67*(9), 939–945.

Blair, L. A. ., & Marshall, J. (1997). IGF-1 Modulates N and L Calcium Channels in a PI 3-Kinase-Dependent Manner. *Neuron*, *19*(2), 421–429. https://doi.org/10.1016/S0896-6273(00)80950-2

Blanchard, R. J., Blanchard, D. C., Agullana, R., & Weiss, S. M. (1991). Twenty-two kHz alarm cries to presentation of a predator, by laboratory rats living in visible burrow systems. *Physiology & Behavior*, *50*(5), 967–972.

Blumberg, M. S., & Sokoloff, G. (2001). Do infant rats cry? *Psychological Review*, *108*(1), 83–95.

Bolton, M. M., Heaney, C. F., Sabbagh, J. J., Murtishaw, A. S., Magcalas, C. M., & Kinney, J. W.

(2012). Deficits in emotional learning and memory in an animal model of schizophrenia. *Behavioural Brain Research*, *233*(1), 35–44.

Borta, A., Wöhr, M., & Schwarting, R. K. W. (2006). Rat ultrasonic vocalization in aversively motivated situations and the role of individual differences in anxiety-related behavior. *Behavioural Brain Research, 166(2),* 271-280. https://doi.org/10.1016/j.bbr.2005.08.009

Bradbury, J. W., & Vehrencamp, S. L. (2011). Conflict Resolution. In *Principles of Animal Communication* (2nd ed., pp. 421–467). Sunderland, Massachusetts: Sinauer Associates, Inc.

Brenes, J. C., Lackinger, M., Höglinger, G. U., Schratt, G., Schwarting, R. K. W., & Wöhr, M. (2016). Differential effects of social and physical environmental enrichment on brain plasticity, cognition, and ultrasonic communication in rats. *Journal of Comparative Neurology*, *524*(8), 1586–1607. https://doi.org/10.1002/cne.23842

Browning, J. R., Browning, D. A., Maxwell, A. O., Dong, Y., Jansen, H. T., Panksepp, J., & Sorg, B. A. (2011). Positive affective vocalizations during cocaine and sucrose self-administration: A model for spontaneous drug desire in rats. *Neuropharmacology*, *61*(1–2), 268–275. https://doi.org/10.1016/j.neuropharm.2011.04.012

Brudzynski, S. M. (2013). Ethotransmission: Communication of emotional states through ultrasonic vocalization in rats. *Current Opinion in Neurobiology, 23(3),* 310-317.

Brudzynski, S. M. (2015). Pharmacology of Ultrasonic Vocalizations in adult Rats: Significance, Call Classification and Neural Substrate. *Current Neuropharmacology*, *13*(2), 180–192. https://doi.org/10.2174/1570159X13999150210141444

Brudzynski, S. M., & Pniak, A. (2002). Social contacts and production of 50-kHz short ultrasonic calls in adult rats. *Journal of Comparative Psychology, 116(1),* 73-82.

Brunelli, S. A., & Hofer, M. A. (2001). Selective Breeding for an Infantile Phenotype (Isolation Calling). In: Blass E.M. (Eds) Developmental Psychobiology, Handbook of Behavioral Neurobiology, vol 13. Springer, Boston, MA.

Burgdorf, J., Colechio, M. E., Stanton, P. K., & Panksepp, J. (2017). Positive emotional learning Induces resilience to depression: A role for NMDA receptor-mediated synaptic plasiticity. *Current Neuropharmacology*, *15*(1), 3–10.

Burgdorf, J., Knutson, B., & Panksepp, J. (2000). Anticipation of rewarding electrical brain stimulation evokes ultrasonic vocalization in rats. *Behavioral Neuroscience*, *114*(2), 320–327. https://doi.org/10.1037//0735-7044.114.2.320

Burgdorf, J., Knutson, B., Panksepp, J., & Ikemoto, S. (2001). Nucleus accumbens amphetamine microinjections unconditionally elicit 50-kHz ultrasonic vocalizations in rats. *Behavioral Neuroscience*, *115*(4), 940–944.

Burgdorf, J., Kroes, R. A., Moskal, J. R., Pfaus, J. G., Brudzynski, S. M., & Panksepp, J. (2008). Ultrasonic vocalizations of rats (*Rattus norvegicus*) during mating, play, and aggression: Behavioral concomitants, relationship to reward, and self-administration of playback.. *Journal of Comparative Psychology*, *122*(4), 357–367.

Burgdorf, J., Moskal, J. R., Brudzynski, S. M., & Panksepp, J. (2013). Rats selectively bred for low levels of play-induced 50kHz vocalizations as a model for Autism Spectrum Disorders: A role for NMDA receptors. *Behavioural Brain Research*, *251*, 18–24. https://doi.org/10.1016/j.bbr.2013.04.022

Burgdorf, J., & Panksepp, J. (2001). Tickling induces reward in adolescent rats. *Physiology & Behavior*, *72*(1–2), 167–173. https://doi.org/10.1016/S0031-9384(00)00411-X

Burgdorf, J., & Panksepp, J. (2006). The neurobiology of positive emotions. *Neuroscience & Biobehavioral Reviews*, *30*(2), 173–187.

Burgdorf, J., Panksepp, J., Brudzynski, S. M., Kroes, R., & Moskal, J. R. (2005). Breeding for 50-kHz Positive Affective Vocalization in Rats. *Behavior Genetics*, *35*(1), 67–72.

Burgdorf, J., Panksepp, J., & Moskal, J. R. (2011). Frequency-modulated 50 kHz ultrasonic vocalizations: a tool for uncovering the molecular substrates of positive affect. *Neuroscience & Biobehavioral Reviews*, *35*(9), 1831–1836.

Burgdorf, J., Wood, P. L., Kroes, R. A., Moskal, J. R., & Panksepp, J. (2007). Neurobiology of 50-kHz ultrasonic vocalizations in rats: Electrode mapping, lesion, and pharmacology studies. *Behavioural Brain Research*, *182*(2), 274–283.

Burke, C. J., Kisko, T. M., Pellis, S. M., & Euston, D. R. (2017). Avoiding escalation from play to aggression in adult male rats: The role of ultrasonic calls. *Behavioural Processes*, *144*, 72–81. https://doi.org/10.1016/j.beproc.2017.09.014

Burke, C. J., Kisko, T. M., Swiftwolfe, H., Pellis, S. M., & Euston, D. R. (2017). Specific 50-kHz vocalizations are tightly linked to particular types of behavior in juvenile rats anticipating play. *PLOS ONE*, *12*(5), e0175841.

Burman, O. H. P., Ilyat, A., Jones, G., & Mendl, M. (2007). Ultrasonic vocalizations as indicators of welfare for laboratory rats (Rattus norvegicus). *Applied Animal Behaviour Science*, *104*(1–2), 116–129. https://doi.org/10.1016/j.applanim.2006.04.028

Burns, T., & Patrick, D. (2007). Social functioning as an outcome measure in schizophrenia studies. *Acta Psychiatrica Scandinavica*, *116*(6), 403–418.

Can, A., Dao, D. T., Arad, M., Terrillion, C. E., Piantadosi, S. C., & Gould, T. D. (2012). The Mouse Forced Swim Test. *Journal of Visualized Experiments : JoVE*, (59), 3638. https://doi.org/10.3791/3638

Cardinal, R. N., Parkinson, J. A., Hall, J., & Everitt, B. J. (2002). Emotion and motivation: the role of the amygdala, ventral striatum, and prefrontal cortex. *Neuroscience & Biobehavioral Reviews*, *26*(3), 321–352. https://doi.org/10.1016/S0149-7634(02)00007-6

Carman, J. S., & Wyatt, R. J. (1979). Calcium: Bivalent cation in the bivalent psychoses. *Biological Psychiatry, 14(2),* 295-336.

Catterall, W. A. (2011). Voltage-gated calcium channels. *Cold Spring Harbor Perspectives in Biology*, *3*(8), a003947.

Cenci, M. A., Whishaw, I. Q., & Schallert, T. (2002). Opinion: Animal models of neurological deficits: how relevant is the rat? *Nature Reviews Neuroscience*, *3*(7), 574–579. https://doi.org/10.1038/nrn877

Chameau, P., Qin, Y., Spijker, S., Smit, G., & Joëls, M. (2007). Glucocorticoids Specifically Enhance L-Type Calcium Current Amplitude and Affect Calcium Channel Subunit Expression in the Mouse Hippocampus. *Journal of Neurophysiology*, *97*(1), 5–14. https://doi.org/10.1152/jn.00821.2006

Charman, T., Jones, C. R. G., Pickles, A., Simonoff, E., Baird, G., & Happé, F. (2011). Defining the cognitive phenotype of autism. *Brain Research*, *1380*, 10–21.

Choong, K., & Shen, R. (2004). Prenatal ethanol exposure alters the postnatal development of the spontaneous electrical activity of dopamine neurons in the ventral tegmental area. *Neuroscience*, *126*(4), 1083–1091.

Christensen, J., Grønborg, T. K., Sørensen, M. J., Schendel, D., Parner, E. T., Pedersen, L. H., & Vestergaard, M. (2013). Prenatal Valproate Exposure and Risk of Autism Spectrum Disorders and Childhood Autism. *JAMA*, *309*(16), 1696-1703.

Cirillo, M. A., & Seidman, L. J. (2003). Verbal declarative memory dysfunction in schizophrenia: from clinical assessment to genetics and brain mechanisms. *Neuropsychology Review*, *13*(2), 43–77.

Consortium, C.-D. G. of the P. G. (2013). Identification of risk loci with shared effects on five major psychiatric disorders: a genome-wide analysis. *Lancet, 981*(9875)*,* 1371-1379.

Craddock, N., & Sklar, P. (2013). Genetics of bipolar disorder. *The Lancet*, *381*(9878), 1654–1662. https://doi.org/10.1016/S0140-6736(13)60855-7

Cryan, J. F., & Holmes, A. (2005). The ascent of mouse: advances in modelling human depression and anxiety. *Nature Reviews Drug Discovery*, *4*(9), 775–790.

Cryan, J. F., & Mombereau, C. (2004). In search of a depressed mouse: utility of models for studying depression-related behavior in genetically modified mice. *Molecular Psychiatry*, *9*(4), 326–357. https://doi.org/10.1038/sj.mp.4001457

Csikszentmihalyi, M., & Hunter, J. (2014). Happiness in everyday life: The uses of experience sampling. *Flow and the Foundations of Positive Psychology*. Springer, Doedrecht. https://doi.org/10.1007/978-94-017-9088-8_6

Czéh, B., & Lucassen, P. J. (2007). What causes the hippocampal volume decrease in depression? *European Archives of Psychiatry and Clinical Neuroscience*, *257*(5), 250–260. https://doi.org/10.1007/s00406-007-0728-0

D'Gama, A. M., Pochareddy, S., Li, M., Jamuar, S. S., Reiff, R. E., Lam, A.-T. N., … Walsh, C. A. (2015). Targeted DNA Sequencing from Autism Spectrum Disorder Brains Implicates Multiple Genetic Mechanisms. *Neuron*, *88*(5), 910–917.

Daenen, E. W. P. ., Wolterink, G., Gerrits, M. A. F. ., & Van Ree, J. M. (2002). The effects of neonatal lesions in the amygdala or ventral hippocampus on social behaviour later in life.

Behavioural Brain Research, *136*(2), 571–582. https://doi.org/10.1016/S0166-4328(02)00223-1

Dao, D. T., Mahon, P. B., Cai, X., Kovacsics, C. E., Blackwell, R. A., Arad, M., … Gould, T. D. (2010). Mood disorder susceptibility gene *CACNA1C* modifies mood-related behaviors in mice and interacts with sex to influence behavior in mice and diagnosis in humans. *Biological Psychiatry, 68*(9), 801-810.

Davis, S. E., & Bauer, E. P. (2012). L-Type Voltage-Gated Calcium Channels in the Basolateral Amygdala Are Necessary for Fear Extinction. *Journal of Neuroscience*, *32*(39), 13582–13586. https://doi.org/10.1523/JNEUROSCI.0809-12.2012

Dedic, N., Pohlmann, M. L., Richter, J. S., Mehta, D., Czamara, D., Metzger, M. W., … Deussing, J. M. (2017, July 11). Cross-disorder risk gene *CACNA1C* differentially modulates susceptibility to psychiatric disorders during development and adulthood. *Moecular Psychiatry*, 23(3), 533-543. http://dx.doi.org/10.1038/mp.2017.133

Dere, E., Pause, B. M., & Pietrowsky, R. (2010). Emotion and episodic memory in neuropsychiatric disorders. *Behavioural Brain Research*, *215*(2), 162–171.

Dichter, G. S., Damiano, C. A., & Allen, J. A. (2012). Reward circuitry dysfunction in psychiatric and neurodevelopmental disorders and genetic syndromes: animal models and clinical findings. *Journal of Neurodevelopmental Disorders*, *4*(1), 19.

Dietsche, B., Backes, H., Laneri, D., Weikert, T., Witt, S. H., Rietschel, M., … Krug, A. (2014). The impact of a *CACNA1C* gene polymorphism on learning and hippocampal formation in healthy individuals: A diffusion tensor imaging study. *NeuroImage*, *89*, 256–261.

Dima, D., Jogia, J., Collier, D., Vassos, E., Burdick, K. E., & Frangou, S. (2013). Independent Modulation of Engagement and Connectivity of the Facial Network During Affect Processing by *CACNA1C* and *ANK3* Risk Genes for Bipolar Disorder. *JAMA Psychiatry*, *70*(12), 1303. https://doi.org/10.1001/jamapsychiatry.2013.2099

Dodell-Feder, D., Tully, L. M., Lincoln, S. H., & Hooker, C. I. (2014). The neural basis of theory of mind and its relationship to social functioning and social anhedonia in individuals with schizophrenia. *NeuroImage: Clinical*, *4*, 154–163.

Dolphin, A. C. (2009, June). Calcium channel diversity: multiple roles of calcium channel subunits. *Current Opinion in Neurobiology*. https://doi.org/10.1016/j.conb.2009.06.006

Drevets, W. C., Price, J. L., & Furey, M. L. (2008). Brain structural and functional abnormalities in mood disorders: implications for neurocircuitry models of depression. *Brain Structure and Function*, *213*(1–2), 93–118. https://doi.org/10.1007/s00429-008-0189-x

Dubovsky, S. L., Thomas, M., Hijazi, A., & Murphy, J. (1994). Intracellular calcium signalling in peripheral cells of patients with bipolar affective disorder. *European Archives of Psychiatry and Clinical Neuroscience*, *243*(5), 229–234.

Ellenbroek, B., & Youn, J. (2016). Rodent models in neuroscience research: is it a rat race? *Disease Models & Mechanisms*, *9*(10), 1079–1087. https://doi.org/10.1242/dmm.026120

Elliott, R., Sahakian, B. J., McKay, A. P., Herrod, J. J., Robbins, T. W., & Paykel, E. S. (1996). Neuropsychological impairments in unipolar depression: the influence of perceived failure on subsequent performance. *Psychological Medicine*, *26*(5), 975-989.

Emamghoreishi, M., Schlichter, L., Li, P. P., Parikh, S., Sen, J., Kamble, A., & Warsh, J. J. (1997). High intracellular calcium concentrations in transformed lymphoblasts from subjects with bipolar I disorder. *American Journal of Psychiatry*, *154*(7), 976–982. https://doi.org/10.1176/ajp.154.7.976

Endres, T., Widmann, K., & Fendt, M. (2007). Are rats predisposed to learn 22 kHz calls as danger-predicting signals? *Behavioural Brain Research*, *185*(2), 69–75.

Engelhardt, K. A., Fuchs, E., Schwarting, R. K. W., & Wöhr, M. (2017). Effects of amphetamine on pro-social ultrasonic communication in juvenile rats: Implications for mania models. *European Neuropsychopharmacology*, *27*(3), 261–273.

Ennaceur, A., & Delacour, J. (1988). A new one-trial test for neurobiological studies of memory in rats. 1: Behavioral data. *Behavioural Brain Research*, *31*(1), 47–59.

Erk, S., Meyer-Lindenberg, A., Linden, D. E. J., Lancaster, T., Mohnke, S., Grimm, O., … Walter, H. (2014). Replication of brain function effects of a genome-wide supported psychiatric risk variant in the *CACNA1C* gene and new multi-locus effects. *NeuroImage*, *94*, 147–154. https://doi.org/10.1016/j.neuroimage.2014.03.007

Erk, S., Meyer-Lindenberg, A., Schmierer, P., Mohnke, S., Grimm, O., Garbusow, M., … Walter, H. (2014). Hippocampal and Frontolimbic Function as Intermediate Phenotype for Psychosis: Evidence from Healthy Relatives and a Common Risk Variant in *CACNA1C*. *Biological Psychiatry*, *76*(6), 466–475.

Erk, S., Meyer-Lindenberg, A., Schnell, K., Opitz von Boberfeld, C., Esslinger, C., Kirsch, P., … Walter, H. (2010). Brain function in carriers of a genome-wide supported bipolar disorder variant. *Archives of General Psychiatry*, *67*(8), 803–811.

Escudero, I., & Johnstone, M. (2014). Genetics of Schizophrenia. *Current Psychiatry Reports, 16,* 502.

Fendt, M., & Fanselow, M. S. (1999). The neuroanatomical and neurochemical basis of conditioned fear. *Neuroscience and Biobehavioral Reviews*, *23*(5), 743–760. https://doi.org/10.1016/S0149-7634(99)00016-0

Ferreira, M. A., O'Donovan, M. C., Meng, Y. A., Jones, I. R., Ruderfer, D. M., Jones, L., … Gill, M. (2008). Collaborative genome-wide association analysis supports a role for *ANK3* and *CACNA1C* in bipolar disorder. *Nature Genetics*, *40(9)*, 1056-1058.

Flinn, M. V, Nepomnaschy, P. A., Muehlenbein, M. P., & Ponzi, D. (2011). Evolutionary functions of early social modulation of hypothalamic-pituitary-adrenal axis development in humans. *Neuroscience & Biobehavioral Reviews*, *35*(7), 1611–1629.

Fone, K. C. F., & Porkess, M. V. (2008). Behavioural and neurochemical effects of post-weaning social isolation in rodents—Relevance to developmental neuropsychiatric disorders. *Neuroscience & Biobehavioral Reviews*, *32*(6), 1087–1102.

Frazier, T. W., Youngstrom, E. A., Frankel, B. A., Zunta-Soares, G. B., Sanches, M., Escamilla, M., … Soares, J. C. (2014). Candidate gene associations with mood disorder, cognitive vulnerability, and fronto-limbic volumes. *Brain and Behavior*, *4*(3), 418–430. https://doi.org/10.1002/brb3.226

Gershon, E. S., Grennan, K., Busnello, J., Badner, J. A., Ovsiew, F., Memon, S., … Liu, C. (2014). A rare mutation of *CACNA1C* in a patient with bipolar disorder, and decreased gene expression associated with a bipolar-associated common SNP of *CACNA1C* in brain. *Molecular Psychiatry, 19,* 890-894. https://doi.org/10.1038/mp.2013.107

Geschwind, D. H. (2011). Genetics of autism spectrum disorders. *Trends in Cognitive Sciences*. https://doi.org/10.1016/j.tics.2011.07.003

Geurts, A. M., Cost, G. J., Freyvert, Y., Zeitler, B., Miller, J. C., Choi, V. M., … Buelow, R. (2009). Knockout Rats via Embryo Microinjection of Zinc-Finger Nucleases. *Science*, *325*(5939), 433.

Gil-da-Costa, R., Braun, A., Lopes, M., Hauser, M. D., Carson, R. E., Herscovitch, P., & Martin, A. (2004). Toward an evolutionary perspective on conceptual representation: Species-specific calls activate visual and affective processing systems in the macaque. *Proceedings of the National Academy of Sciences*, *101*(50), 17516–17521.

Gitlin, M. J., Swendsen, J., Heller, T. L., & Hammen, C. (1995). Relapse and impairment in bipolar disorder. *American Journal of Psychiatry*, *152*(11), 1635–1640. https://doi.org/10.1176/ajp.152.11.1635

Goldberg, J. F., & Harrow, M. (2004). Consistency of remission and outcome in bipolar and unipolar mood disorders: a 10-year prospective follow-up. *Journal of Affective Disorders*, *81*(2), 123–131. https://doi.org/10.1016/S0165-0327(03)00161-7

Graham, L. K., Yoon, T., Lee, H. J., & Kim, J. J. (2009). Strain and sex differences in fear conditioning: 22 kHz ultrasonic vocalizations and freezing in rats. *Psychology & Neuroscience*, *2*(2), 219–225. https://doi.org/10.3922/j.psns.2009.2.015

Grayson, B., Leger, M., Piercy, C., Adamson, L., Harte, M., & Neill, J. C. (2015). Assessment of disease-related cognitive impairments using the novel object recognition (NOR) task in rodents. *Behavioural Brain Research*, *285*, 176–193.

Green, E. K., Grozeva, D., Jones, I., Jones, L., Kirov, G., Caesar, S., … Craddock, N. (2010). The bipolar disorder risk allele at *CACNA1C* also confers risk of recurrent major depression and of schizophrenia. *Molecular Psychiatry, 15*, 1016-1022.

Green, M. F. (1996). What are the functional consequences of neurocognitive deficits in schizophrenia? *American Journal of Psychiatry*, *153*(3), 321–330.

Gruendel, A. D., & Arnold, W. J. (1969). Effects of early social deprivation on reproductive behavior of male rats. *Journal of Comparative and Physiological Psychology, 67*(1), 123-128.

Gunnar, M. R., Frenn, K., Wewerka, S. S., & Van Ryzin, M. J. (2009). Moderate versus severe early life stress: Associations with stress reactivity and regulation in 10–12-year-old

children. *Psychoneuroendocrinology*, *34*(1), 62–75.

Hall, F. S. (1998). Social deprivation of neonatal, adolescent, and adult rats has distinct neurochemical and behavioral consequences. *Critical Reviews in Neurobiology*, *12*(1–2), 129-162.

Hammar, Å., & Årdal, G. (2009). Cognitive functioning in major depression – a summary. *Frontiers in Human Neuroscience*, *3*, 26. https://doi.org/10.3389/neuro.09.026.2009

Hård, E., & Larsson, K. (1968). Dependence of Adult Mating Behavior in Male Rats on the Presence of Littermates in Infancy. *Brain, Behavior and Evolution*, *1*(5), 405–419. https://www.karger.com/DOI/10.1159/000125517

Harmon, K. M., Cromwell, H. C., Burgdorf, J., Moskal, J. R., Brudzynski, S. M., Kroes, R. A., & Panksepp, J. (2008). Rats selectively bred for low levels of 50 kHz ultrasonic vocalizations exhibit alterations in early social motivation. *Developmental Psychobiology*, *50*(4), 322–331. https://doi.org/10.1002/dev.20294

Harvey, P. D., Lombardi, J., Leibman, M., White, L., Parrella, M., Powchik, P., & Davidson, M. (1996). Cognitive impairment and negative symptoms in geriatric chronic schizophrenic patients: a follow-up study. *Schizophrenia Research*, *22*(3), 223–231. https://doi.org/10.1016/S0920-9964(96)00075-8

Hennessy, M. B., & Weinberg, J. (1990). Adrenocortical activity during conditions of brief social separation in preweaning rats. *Behavioral and Neural Biology*, *54*(1), 42–55. https://doi.org/10.1016/0163-1047(90)91231-Y

Henry, C., Kabbaj, M., Simon, H., Le Moal, M., & Maccari, S. (1994). Prenatal Stress Increases the Hypothalamo-Pituitary-Adrenal Axis Response in Young and Adult Rats. *Journal of Neuroendocrinology*, *6*(3), 341–345.

Heyes, S., Pratt, W. S., Rees, E., Dahimene, S., Ferron, L., Owen, M. J., & Dolphin, A. C. (2015). Genetic disruption of voltage - gated calcium channels in psychiatric and neurological disorders. *Progress in Neurobiology, 134,* 36-54.

Himmler, B. T., Kisko, T. M., Euston, D. R., Kolb, B., & Pellis, S. M. (2014). Are 50-kHz calls used as play signals in the playful interactions of rats? I. Evidence from the timing and context of their use. *Behavioural Processes*, *106*, 60–66.

Himmler, S. M., Modlinska, K., Stryjek, R., Himmler, B. T., Pisula, W., & Pellis, S. M. (2014). Domestication and diversification: A comparative analysis of the play fighting of the Brown Norway, Sprague-Dawley, and Wistar laboratory strains of (Rattus norvegicus). *Journal of Comparative Psychology*, *128*(3), 318–327. https://doi.org/10.1037/a0036104

Hirschfeld, R. M. A., Montgomery, S. A., Keller, M. B., Kasper, S., Schatzberg, A. F., Moller, H.-J., … Bourgeois, M. (2000). Social Functioning in Depression. *The Journal of Clinical Psychiatry*, *61*(4), 268–275. https://doi.org/10.4088/JCP.v61n0405

Hofer, M. A. (1996). Multiple regulators of ultrasonic vocalization in the infant rat. *Psychoneuroendocrinology*, *21*(2), 203–217.

Hofer, M. A., Shair, H. N., & Brunelli, S. A. (2002). Ultrasonic Vocalizations in Rat and Mouse Pups. In *Current Protocols in Neuroscience*. Hoboken, NJ, USA: John Wiley & Sons, Inc. https://doi.org/10.1002/0471142301.ns0814s17

Hol, T., Van Den Berg, C. L., Van Ree, J. M., & Spruijt, B. M. (1999). Isolation during the play period in infancy decreases adult social interactions in rats. *Behavioural Brain Research*, *100*(1–2), 91–97. https://doi.org/10.1016/S0166-4328(98)00116-8

Homberg, J. R., Schiepers, O. J. G., Schoffelmeer, A. N. M., Cuppen, E., & Vanderschuren, L. J. (2007). Acute and constitutive increases in central serotonin levels reduce social play behaviour in peri-adolescent rats. *Psychopharmacology*, *195*(2), 175–182. https://doi.org/10.1007/s00213-007-0895-8

Homberg, J. R., Wöhr, M., & Alenina, N. (2017). Comeback of the Rat in Biomedical Research. *ACS Chemical Neuroscience*, *8*(5), 900–903.

Hori, M., Shimoju, R., Tokunaga, R., Ohkubo, M., Miyabe, S., Ohnishi, J., … Kurosawa, M. (2013). Tickling increases dopamine release in the nucleus accumbens and 50 kHz ultrasonic vocalizations in adolescent rats. *NeuroReport*, *24*(5), 241-245.

Hutson, P. H., Tarazi, F. I., Madhoo, M., Slawecki, C., & Patkar, A. A. (2014). Preclinical pharmacology of amphetamine: Implications for the treatment of neuropsychiatric disorders. *Pharmacology and Therapeutics*, *143*(3), 253–264.

Insel, T. R., & Winslow, J. T. (1991). Central administration of oxytocin modulates the infant rats response to social isolation. *European Journal of Pharmacology*, *203*(1), 149–152.

Jeon, D., Kim, S., Chetana, M., Jo, D., Earl Ruley, H., Lin, S.-Y., … Shin, H.-S. (2010). Observational fear learning involves affective pain system and Cav1.2 Ca2+ channels in ACC. *Nature Neuroscience*, *13*(4), 482–488. https://doi.org/10.1038/nn.2504

Jessen, H. M., Kolodkin, M. H., Bychowski, M. E., Auger, C. J., & Auger, A. P. (2010). The Nuclear Receptor Corepressor Has Organizational Effects within the Developing Amygdala on Juvenile Social Play and Anxiety-Like Behavior. *Endocrinology*, *151*(3), 1212–1220. https://doi.org/10.1210/en.2009-0594

Jimerson, D. C., Post, R. M., Carman, J. S., van Kammen, D. P., Wood, J. H., Goodwin, F. K., & Bunney, W. E. (1979). CSF calcium: Clinical correlates in affective illness and schizophrenia. *Biological Psychiatry, 14*(1), 37-51.

Jogia, J., Ruberto, G., Lelli-Chiesa, G., Vassos, E., Maierú, M., Tatarelli, R., … Frangou, S. (2011). The impact of the *CACNA1C* gene polymorphism on frontolimbic function in bipolar disorder. *Molecular Psychiatry*, *16*(11), 1070–1071.

Joyce, E. M., & Roiser, J. P. (2007). Cognitive heterogeneity in schizophrenia. *Current Opinion in Psychiatry*, *20*(3), 268–272. https://doi.org/10.1097/YCO.0b013e3280ba4975

Kabir, Z. D., Che, A., Fischer, D. K., Rice, R. C., Rizzo, B. K., Byrne, M., … Rajadhyaksha, A. M. (2017). Rescue of impaired sociability and anxiety-like behavior in adult *CACNA1C*-deficient mice by pharmacologically targeting eIF2α. *Molecular Psychiatry*, *22*(8), 1096–1109. https://doi.org/10.1038/mp.2017.124

Kabir, Z. D., Lee, A. S., & Rajadhyaksha, A. M. (2016). L-type Ca2+ channels in mood, cognition and addiction: integrating human and rodent studies with a focus on behavioural endophenotypes. *The Journal of Physiology*, *594*(20), 5823–5837.

Kabitzke, P., Brunner, D., He, D., Fazio, P. A., Cox, K., Sutphen, J., … Luo Clayton, A. (2017). Comprehensive Analysis of Two Shank3 and the *CACNA1C* Mouse Models of Autism Spectrum Disorder. *Genes, Brain, and Behavior*, 17(1), 1–36.

Karst, H., Nair, S., Velzing, E., Rumpff-van Essen, L., Slagter, E., Shinnick-Gallagher, P., & Joëls, M. (2002). Glucocorticoids alter calcium conductances and calcium channel subunit expression in basolateral amygdala neurons. *European Journal of Neuroscience*, *16*(6), 1083–1089. https://doi.org/10.1046/j.1460-9568.2002.02172.x

Keeler, J. F., & Robbins, T. W. (2011). Translating cognition from animals to humans. *Biochemical Pharmacology*, *81*(12), 1356–1366.

Kendler, K. S., Gatz, M., Gardner, C. O., & Pedersen, N. L. (2006). A Swedish National Twin Study of Lifetime Major Depression. *American Journal of Psychiatry*, *1631*(163), 109–114.

Kendler, K. S., & Prescott, C. A. (1999). A Population-Based Twin Study of Lifetime Major Depression in Men and Women. *Archives of General Psychiatry*, *56*(1), 39. https://doi.org/10.1001/archpsyc.56.1.39

Kessler, R. C., Chiu, W. T., Demler, O., & Walters, E. E. (2005). Prevalence, Severity, and Comorbidity of Twelve-month DSM-IV Disorders in the National Comorbidity Survey Replication (NCS-R). *Archives of General Psychiatry*, *62*(6), 617–627. https://doi.org/10.1001/archpsyc.62.6.617

Kim, E. J., Kim, E. S., Covey, E., & Kim, J. J. (2010). Social Transmission of Fear in Rats: The Role of 22-kHz Ultrasonic Distress Vocalization. *PLoS ONE*, *5*(12), e15077. https://doi.org/10.1371/journal.pone.0015077

Kisko, T. M., Euston, D. R., & Pellis, S. M. (2015). Are 50-khz calls used as play signals in the playful interactions of rats? III. The effects of devocalization on play with unfamiliar partners as juveniles and as adults. *Behavioural Processes*, *113,* 113-121.

Kisko, T. M., Himmler, B. T., Himmler, S. M., Euston, D. R., & Pellis, S. M. (2015). Are 50-kHz calls used as play signals in the playful interactions of rats? II. Evidence from the effects of devocalization. *Behavioural Processes*, *111,* 25-33.

Kisko, T. M., Wöhr, M., Pellis, V. C., & Pellis, S. M. (2015). From Play to Aggression: High-Frequency 50-kHz Ultrasonic Vocalizations as Play and Appeasement Signals in Rats. In M. Wöhr & S. Krach (Eds.), *Social Behavior from Rodents to Humans: Neural Foundations and Clinical Implications* (pp. 91–108). Switzerland: Springer International Publishing.

Knutson, B., Burgdorf, J., & Panksepp, J. (1998). Anticipation of play elicits high-frequency ultrasonic vocalizations in young rats. *Journal of Comparative Psychology*, *112*(1), 65–73. https://doi.org/10.1037/0735-7036.112.1.65

Knutson, B., Burgdorf, J., & Panksepp, J. (1999). High-frequency ultrasonic vocalizations index conditioned pharmacological reward in rats. *Physiology and Behavior, 66*(4), 639-643. https://doi.org/10.1016/S0031-9384(98)00337-0

Knutson, B., Burgdorf, J., & Panksepp, J. (2002). Ultrasonic vocalizations as indices of affective states in rats. *Psychological Bulletin, 128*(6), 961-977.

Kolb, B. (1984). Functions of the frontal cortex of the rat: A comparative review. *Brain Research Reviews*, *8*(1), 65–98. https://doi.org/10.1016/0165-0173(84)90018-3

Krug, A., Nieratschker, V., Markov, V., Krach, S., Jansen, A., Zerres, K., … Kircher, T. (2010). Effect of *CACNA1C* rs1006737 on neural correlates of verbal fluency in healthy individuals. *NeuroImage*. https://doi.org/10.1016/j.neuroimage.2009.09.028

Krug, A., Witt, S. H., Backes, H., Dietsche, B., Nieratschker, V., Shah, N. J., … Kircher, T. (2014). A genome-wide supported variant in *CACNA1C* influences hippocampal activation during episodic memory encoding and retrieval. *European Archives of Psychiatry and Clinical Neuroscience, 264*(2), 103-110. https://doi.org/10.1007/s00406-013-0428-x

Kuehner, C. (2003). Gender differences in unipolar depression: an update of epidemiological findings and possible explanations. *Acta Psychiatrica Scandinavica*, *108*(3), 163–174. https://doi.org/10.1034/j.1600-0447.2003.00204.x

Kuperberg, G., & Heckers, S. (2000). Schizophrenia and cognitive function. *Current Opinion in Neurobiology*, *10*(2), 205–210. https://doi.org/10.1016/S0959-4388(00)00068-4

Kuraoka, K., & Nakamura, K. (2010). Vocalization as a specific trigger of emotional responses. *Handbook of Behavioral Neuroscience*, *19*, 167–175.

Kurian, J. R., Bychowski, M. E., Forbes-Lorman, R. M., Auger, C. J., & Auger, A. P. (2008). Mecp2 Organizes Juvenile Social Behavior in a Sex-Specific Manner. *Journal of Neuroscience*, *28*(28), 7137–7142. https://doi.org/10.1523/JNEUROSCI.1345-08.2008

Kurtz, M. (2005). Neurocognitive impairment across the lifespan in schizophrenia: an update. *Schizophrenia Research*, *74*(1), 15–26. https://doi.org/10.1016/j.schres.2004.07.005

Lai, M.-C., Lombardo, M. V, & Baron-Cohen, S. (2014). Autism. *The Lancet*, *383*(9920), 896–910. https://doi.org/10.1016/S0140-6736(13)61539-1

Lancaster, T. M., Foley, S., Tansey, K. E., Linden, D. E. J., & Caseras, X. (2016). *CACNA1C* risk variant is associated with increased amygdala volume. *European Archives of Psychiatry and Clinical Neuroscience*, *266*(3), 269–275. https://doi.org/10.1007/s00406-015-0609-x

Langwieser, N., Christel, C. J., Kleppisch, T., Hofmann, F., Wotjak, C. T., & Moosmang, S. (2010). Homeostatic Switch in Hebbian Plasticity and Fear Learning after Sustained Loss of Cav1.2 Calcium Channels. *Journal of Neuroscience*, *30*(25), 8367–8375.

Lapiz, M. D. S., Fulford, A., Muchimapura, S., Mason, R., Parker, T., & Marsden, C. A. (2003). Influence of Postweaning Social Isolation in the Rat on Brain Development, Conditioned Behavior, and Neurotransmission. *Neuroscience and Behavioral Physiology*, *33*(1), 13–29. https://doi.org/10.1023/A:1021171129766

Lecourtier, L., & Kelly, P. H. (2007). A conductor hidden in the orchestra? Role of the habenular complex in monoamine transmission and cognition. *Neuroscience and Biobehavioral Reviews. 31*(5), 658-672. https://doi.org/10.1016/j.neubiorev.2007.01.004

Lee, A. S., De Jesus-Cortes, H., Kabir, Z. D., Knobbe, W., Orr, M., Burgdorf, C., … Pieper, A. A. (2016). The Neuropsychiatric Disease-Associated Gene *CACNA1C* Mediates Survival of Young Hippocampal Neurons. *eNeuro*, *3*(2).

Lee, A. S., Ra, S., Rajadhyaksha, A. M., Britt, J. K., De Jesus-Cortes, H., Gonzales, K. L., … Rajadhyaksha, A. M. (2012). Forebrain elimination of *CACNA1C* mediates anxiety-like behavior in mice. *Molecular Psychiatry*, *17*(11), 1054–1055.

Lee Duckworth, A., Steen, T. A., & Seligman, M. E. P. (2005). Positive Psychology in Clinical Practice. *Annual Review of Clinical Psychology*, *1*(1), 629–651.

Lehner, M. H., Taracha, E., Kaniuga, E., Wisłowska-Stanek, A., Wróbel, J., Sobolewska, A., … Płaźnik, A. (2014). High-anxiety rats are less sensitive to the rewarding affects of amphetamine on 50kHz USV. *Behavioural Brain Research, 275,* 234-242.

Lenz, K. M., Nugent, B. M., & McCarthy, M. M. (2012). Sexual Differentiation of the Rodent Brain: Dogma and Beyond. *Frontiers in Neuroscience*, *6*, 26.

Lépine, J.-P., & Briley, M. (2011). The increasing burden of depression. *Neuropsychiatric Disease and Treatment*, *7*(Suppl 1), 3–7. https://doi.org/10.2147/NDT.S19617

Levine, J., Stein, D., Rapoport, A., & Kurtzman, L. (1999). High Serum and Cerebrospinal Fluid Ca/Mg Ratio in Recently Hospitalized Acutely Depressed Patients. *Neuropsychobiology*, *39*(2), 63–70. https://doi.org/10.1159/000026562

Lewis, K. P., & Barton, R. A. (2006). Amygdala size and hypothalamus size predict social play frequency in nonhuman primates: A comparative analysis using independent contrasts. *Journal of Comparative Psychology*, *120*(1), 31–37. https://doi.org/10.1037/0735-7036.120.1.31

Li, J., Zhao, L., You, Y., Lu, T., Jia, M., Yu, H., … Wang, L. (2015). Schizophrenia related variants in *CACNA1C* also confer risk of autism. *PLoS ONE, 10*(7), e0133247. https://doi.org/10.1371/journal.pone.0133247

Liao, P., & Soong, T. W. (2010). CaV1.2 channelopathies: from arrhythmias to autism, bipolar disorder, and immunodeficiency. *Pflügers Archiv - European Journal of Physiology*, *460*(2), 353–359. https://doi.org/10.1007/s00424-009-0753-0

Lichtenstein, P., Yip, B. H., Björk, C., Pawitan, Y., Cannon, T. D., Sullivan, P. F., & Hultman, C. M. (2009). Common genetic determinants of schizophrenia and bipolar disorder in Swedish families: a population-based study. *The Lancet*, *373*(9659), 234–239. https://doi.org/10.1016/S0140-6736(09)60072-6

Liu, Y., Blackwood, D. H., Caesar, S., Geus, E. J. C. de, Farmer, A., Ferreira, M. A., … Sullivan, P. F. (2011). Meta-analysis of genome-wide association data of bipolar disorder and major depressive disorder. *Molecular Psychiatry, 16,* 2-4.

Lohoff, F. W. (2010). Overview of the genetics of major depressive disorder. *Current Psychiatry Reports, 12*(6), 539-546. https://doi.org/10.1007/s11920-010-0150-6

Łopuch, S., & Popik, P. (2011). Cooperative Behavior of Laboratory Rats (Rattus norvegicus) in an Instrumental Task. *Journal of Comparative Psychology*, *125*(2), 250–253. https://doi.org/10.1037/a0021532

Lore, R., & Flannelly, K. (1977). Rat societies. *Scientific American*, *236*(5), 106–118. http://www.ncbi.nlm.nih.gov/pubmed/558650

Lore, R., Flannelly, K., & Farina, P. (1976). Ultrasounds produced by rats accompany decreases in intraspecific fighting. *Aggressive Behavior*, *2*(3), 175–181.

Lukas, M., & Wöhr, M. (2015). Endogenous vasopressin, innate anxiety, and the emission of pro-social 50-kHz ultrasonic vocalizations during social play behavior in juvenile rats. *Psychoneuroendocrinology*, *56*, 35–44. https://doi.org/10.1016/j.psyneuen.2015.03.005

Lyons, D. M., Parker, K. J., & Schatzberg, A. F. (2010). Animal models of early life stress: Implications for understanding resilience. *Developmental Psychobiology*, *52*(7), 616–624. https://doi.org/10.1002/dev.20500

Ma, S. T., Maier, E. Y., Ahrens, A. M., Schallert, T., & Duvauchelle, C. L. (2010). Repeated intravenous cocaine experience: Development and escalation of pre-drug anticipatory 50-kHz ultrasonic vocalizations in rats. *Behavioural Brain Research*, *212*(1), 109–114. https://doi.org/10.1016/j.bbr.2010.04.001

Maaswinkel, H., Baars, A.-M., Gispen, W.-H., & Spruijt, B. M. (1996). Roles of the basolateral amygdala and hippocampus in social recognition in rats. *Physiology & Behavior*, *60*(1), 55–63. https://doi.org/10.1016/0031-9384(95)02233-3

Macrì, S., Granstrem, O., Shumilina, M., Antunes Gomes dos Santos, F. J., Berry, A., Saso, L., & Laviola, G. (2009). Resilience and vulnerability are dose-dependently related to neonatal stressors in mice. *Hormones and Behavior*, *56*(4), 391–398.

Mahler, S. V., Moorman, D. E., Feltenstein, M. W., Cox, B. M., Ogburn, K. B., Bachar, M., … See, R. E. (2013). A rodent "self-report" measure of methamphetamine craving? Rat ultrasonic vocalizations during methamphetamine self-administration, extinction, and reinstatement. *Behavioural Brain Research*, *236*, 78–89.

Maier, E. Y., Abdalla, M., Ahrens, A. M., Schallert, T., & Duvauchelle, C. L. (2012). The missing variable: ultrasonic vocalizations reveal hidden sensitization and tolerance-like effects during long-term cocaine administration. *Psychopharmacology*, *219*(4), 1141–1152. https://doi.org/10.1007/s00213-011-2445-7

Maier, E. Y., Ahrens, A. M., Ma, S. T., Schallert, T., & Duvauchelle, C. L. (2010). Cocaine deprivation effect: Cue abstinence over weekends boosts anticipatory 50-kHz ultrasonic vocalizations in rats. *Behavioural Brain Research*, *214*(1), 75–79.

Mallas, E.-J., Carletti, F., Chaddock, C. a, Woolley, J., Picchioni, M. M., Shergill, S. S., … Prata, D. P. (2016). Genome-wide discovered psychosis-risk gene *ZNF804A* impacts on white matter microstructure in health, schizophrenia and bipolar disorder. *PeerJ, 4,* e1570.

https://doi.org/10.7717/peerj.1570

Mällo, T., Matrov, D., Herm, L., Kõiv, K., Eller, M., Rinken, A., & Harro, J. (2007). Tickling-induced 50-kHz ultrasonic vocalization is individually stable and predicts behaviour in tests of anxiety and depression in rats. *Behavioural Brain Research*, *184*(1), 57–71. https://doi.org/10.1016/J.BBR.2007.06.015

Manduca, A., Servadio, M., Damsteegt, R., Campolongo, P., Vanderschuren, L. J., & Trezza, V. (2016). Dopaminergic Neurotransmission in the Nucleus Accumbens Modulates Social Play Behavior in Rats. *Neuropsychopharmacology , 41*(9), 2215–2223. https://doi.org/10.1038/npp.2016.22

Markham, J. A., Taylor, A. R., Taylor, S. B., Bell, D. B., & Koenig, J. I. (2010). Characterization of the Cognitive Impairments Induced by Prenatal Exposure to Stress in the Rat. *Frontiers in Behavioral Neuroscience*, *4,* 173.

McGuffin, P., Rijsdijk, F., Andrew, M., Sham, P., Katz, R., & Cardno, A. (2003). The heritability of bipolar affective disorder and the genetic relationship to unipolar depression. *Archives of General Psychiatry*, *60*(5), 497–502.

McGurk, S. R., & Meltzer, H. Y. (2000). The role of cognition in vocational functioning in schizophrenia. *Schizophrenia Research*, *45*(3), 175–184. https://doi.org/10.1016/S0920-9964(99)00198-X

McKinney, B. C., Sze, W., White, J. A., & Murphy, G. G. (2008). L-type voltage-gated calcium channels in conditioned fear: A genetic and pharmacological analysis. *Learning & Memory*, *15*(5), 326–334. https://doi.org/10.1101/lm.893808

Meaney, M. J., Dodge, A. M., & Beatty, W. W. (1981). Sex-dependent effects of amygdaloid lesions on the social play of prepubertal rats. *Physiology and Behavior*, *26*(3), 467–472. https://doi.org/10.1016/0031-9384(81)90175-X

Meaney, M. J., & Stewart, J. (1981). A descriptive study of social development in the rat (Rattus norvegicus). *Animal Behaviour*, *29*(1), 34–45. https://doi.org/10.1016/S0003-3472(81)80149-2

Meyer, P. J., Ma, S. T., & Robinson, T. E. (2012). A cocaine cue is more preferred and evokes more frequency-modulated 50-kHz ultrasonic vocalizations in rats prone to attribute incentive salience to a food cue. *Psychopharmacology*, *219*(4), 999–1009. https://doi.org/10.1007/s00213-011-2429-7

Millan, M. J., Agid, Y., Brüne, M., Bullmore, E. T., Carter, C. S., Clayton, N. S., … Young, L. J. (2012). Cognitive dysfunction in psychiatric disorders: characteristics, causes and the quest for improved therapy. *Nature Reviews Drug Discovery*, *11*(2), 141–168.

Miller, E. K. (2000). The prefontral cortex and cognitive control. *Nature Reviews Neuroscience*, *1*(1), 59–65. https://doi.org/10.1038/35036228

Monteiro, P., & Feng, G. (2017). SHANK proteins: roles at the synapse and in autism spectrum disorder. *Nature Reviews Neuroscience*, *18*(3), 147–157.

Moosmang, S., Haider, N., Klugbauer, N., Adelsberger, H., Langwieser, N., Müller, J., … Kleppisch, T. (2005). Role of Hippocampal $Ca_v1.2$ Ca^{2+} Channels in NMDA Receptor-Independent Synaptic Plasticity and Spatial Memory. *The Journal of Neuroscience*, *25*(43), 9883-9892. http://www.jneurosci.org/content/25/43/9883.abstract

Morellini, F. (2013). Spatial memory tasks in rodents: what do they model? *Cell and Tissue Research*, *354*(1), 273–286. https://doi.org/10.1007/s00441-013-1668-9

Morley-Fletcher, S., Rea, M., Maccari, S., & Laviola, G. (2003). Environmental enrichment during adolescence reverses the effects of prenatal stress on play behaviour and HPA axis reactivity in rats. *European Journal of Neuroscience*, *18*(12), 3367–3374.

Morris, J. S., Frith, C. D., Perrett, D. I., Rowland, D., Young, A. W., Calder, A. J., & Dolan, R. J. (1996). A differential neural response in the human amygdala to fearful and happy facial expressions. *Nature*, *383*(6603), 812–815. https://doi.org/10.1038/383812a0

Morris, R. G. (1981). Spatial localization does not require the presence of local cues. *Learning and Motivation*, *12*(2), 239–260. https://doi.org/10.1016/0023-9690(81)90020-5

Moskal, J. R., Burgdorf, J., Kroes, R. A., Brudzynski, S. M., & Panksepp, J. (2011). A novel NMDA receptor glycine-site partial agonist, GLYX-13, has therapeutic potential for the treatment of autism. *Neuroscience and Biobehavioral Reviews*, *35*(9), 1982–1988.

Moskvina, V., Craddock, N., Holmans, P., Nikolov, I., Pahwa, J. S., Green, E., … O'Donovan, M. C. (2009). Gene-wide analyses of genome-wide association data sets: evidence for multiple common risk alleles for schizophrenia and bipolar disorder and for overlap in genetic risk. *Molecular Psychiatry*, *14,* 252-260. https://doi.org/10.1038/mp.2008.133

Mu, P., Fuchs, T., Saal, D. B., Sorg, B. A., Dong, Y., & Panksepp, J. (2009). Repeated cocaine exposure induces sensitization of ultrasonic vocalization in rats. *Neuroscience Letters*, *453*(1), 31–35. https://doi.org/10.1016/j.neulet.2009.02.007

Noirot, E. (1968). Ultrasounds in young rodents. II. Changes with age in albino rats. *Animal Behaviour*, *16*(1), 129–134. https://doi.org/10.1016/0003-3472(68)90123-1

Nyegaard, M., Demontis, D., Foldager, L., Hedemand, A., Flint, T. J., Sørensen, K. M., … Børglum, A. D. (2010). *CACNA1C* (rs1006737) is associated with schizophrenia. *Molecular Psychiatry, 15*, 119-121. https://doi.org/10.1038/mp.2009.69

Olesen, K. M., Jessen, H. M., Auger, C. J., & Auger, A. P. (2005). Dopaminergic Activation of Estrogen Receptors in Neonatal Brain Alters Progestin Receptor Expression and Juvenile Social Play Behavior. *Endocrinology*, *146*(9), 3705–3712.

Olton, D. S., & Paras, B. C. (1979). Spatial memory and hippocampal function. *Neuropsychologia*, *17*(6), 669–682. https://doi.org/10.1016/0028-3932(79)90042-3

Olton, D. S., & Samuelson, R. J. (1976). Remembrance of places passed: Spatial memory in rats. *Journal of Experimental Psychology: Animal Behavior Processes*, *2*(2), 97–116.

Opendak, M., Gould, E., & Sullivan, R. (2017). Early life adversity during the infant sensitive period for attachment: Programming of behavioral neurobiology of threat processing and

social behavior. *Developmental Cognitive Neuroscience*, *25*, 145–159.

Ou, X., Crane, D. E., MacIntosh, B. J., Young, L. T., Arnold, P., Ameis, S., & Goldstein, B. I. (2015). *CACNA1C* rs1006737 genotype and bipolar disorder: Focus on intermediate phenotypes and cardiovascular comorbidity. *Neuroscience and Biobehavioral Reviews, 55,* 198-210. https://doi.org/10.1016/j.neubiorev.2015.04.022

Owen, M. J., Sawa, A., & Mortensen, P. B. (2016). Schizophrenia. *Lancet*, *388*(10039), 86–97. https://doi.org/10.1016/S0140-6736(15)01121-6

Owren, M. J., & Rendall, D. (1997). An Affect-Conditioning Model of Nonhuman Primate Vocal Signaling. In D. H. Owings, M. D. Beecher, & N. S. Thompson (Eds.), *Communication* (pp. 299–346). Boston, MA: Springer US. https://doi.org/10.1007/978-1-4899-1745-4_10

Palagi, E., & Paoli, T. (2007). Play in adult bonobos (Pan paniscus): Modality and potential meaning. *American Journal of Physical Anthropology*, *134*(2), 219–225.

Panksepp, J. (1981). The ontogeny of play in rats. *Developmental Psychobiology*, *14*(4), 327–332. https://doi.org/10.1002/dev.420140405

Panksepp, J. (2005). Beyond a Joke: From Animal Laughter to Human Joy? *Science*, *308*(5718), 62-63. http://science.sciencemag.org/content/308/5718/62.abstract

Panksepp, J., & Beatty, W. W. (1980). Social deprivation and play in rats. *Behavioral and Neural Biology*, *30*(2), 197–206. https://doi.org/10.1016/S0163-1047(80)91077-8

Panksepp, J., & Burgdorf, J. (2000). 50-kHz chirping (laughter?) in response to conditioned and unconditioned tickle-induced reward in rats: effects of social housing and genetic variables. *Behavioural Brain Research*, *115*(1), 25–38.

Panksepp, J., & Burgdorf, J. (2003). "Laughing" rats and the evolutionary antecedents of human joy? *Physiology and Behavior*, *79*(3), 533–547. https://doi.org/10.1016/S0031-9384(03)00159-8

Panksepp, J., Gordon, N., & Burgdorf, J. (2002). Empathy and the action-perception resonances of basic socio-emotional systems of the brain. *Behavioral and Brain Sciences*, *25*(1), 43-44. https://doi.org/10.1017/S0140525X0247001X

Panksepp, J., Siviy, S., & Normansell, L. (1984). The psychobiology of play: Theoretical and methodological perspectives. *Neuroscience & Biobehavioral Reviews*, *8*(4), 465–492.

Parsana, A. J., Li, N., & Brown, T. H. (2012). Positive and negative ultrasonic social signals elicit opposing firing patterns in rat amygdala. *Behavioural Brain Research*, *226*(1), 77–86. https://doi.org/10.1016/j.bbr.2011.08.040

Parsana, A. J., Moran, E. E., & Brown, T. H. (2012). Rats learn to freeze to 22-kHz ultrasonic vocalizations through autoconditioning. *Behavioural Brain Research*, *232*(2), 395–399.

Pasparakis, E., Koiliari, E., Zouraraki, C., Tsapakis, E. M., Roussos, P., Giakoumaki, S. G., & Bitsios, P. (2015). The effects of the *CACNA1C* rs1006737 A/G on affective startle modulation in healthy males. *European Psychiatry, 30*(4), 492-498.

Paulus, F. M., Bedenbender, J., Krach, S., Pyka, M., Krug, A., Sommer, J., … Jansen, A. (2014). Association of rs1006737 in *CACNA1C* with alterations in prefrontal activation and fronto-hippocampal connectivity. *Human Brain Mapping*, *35*(4), 1190–1200. https://doi.org/10.1002/hbm.22244

Pellis, S. M. (1988). Agonistic versus amicable targets of attack and defense: Consequences for the origin, function, and descriptive classification of play-fighting. *Aggressive Behavior*, *14*(2), 85–104.

Pellis, S. M. (2002). Sex Differences in Play Fighting Revisited: Traditional and Nontraditional Mechanisms of Sexual Differentiation in Rats. *Archives of Sexual Behavior*, *31*(1), 17–26. https://doi.org/10.1023/A:1014070916047

Pellis, S. M., Castañeda, E., McKenna, M. M., Tran-Nguyen, L. T. L., & Whishaw, I. Q. (1993). The role of the striatum in organizing sequences of play fighting in neonatally dopamine-depleted rats. *Neuroscience Letters*, *158*(1), 13–15.

Pellis, S. M., & Pellis, V. C. (1987). Play-fighting differs from serious fighting in both target of attack and tactics of fighting in the laboratory ratRattus norvegicus. *Aggressive Behavior*, *13*(4), 227–242.

Pellis, S. M., & Pellis, V. C. (1991). Attack and defense during play fighting appear to be motivationally independent behaviors in muroid rodents. *The Psychological Record*, *41*(2), 175–184.

Pellis, S. M., & Pellis, V. C. (1997). The Prejuvenile Onset of Play Fighting in Laboratory Rats (*Rattus norvegicu*s). *Developmental Psychobiology*, *31*(3), 193–205.

Pellis, S. M., & Pellis, V. C. (2009). *The playful brain: venturing to the limits of neuroscience*. Oxford, England, Oneworld Publications.

Pellis, S. M., Pellis, V. C., & Whishaw, I. Q. (1992). The role of the cortex in play fighting by rats: Developmental and evolutionary implications. *Brain, Behavior and Evolution*, *39*(5), 270–284.

Pereira, M., Andreatini, R., Schwarting, R. K. W., & Brenes, J. C. (2014). Amphetamine-induced appetitive 50-kHz calls in rats: a marker of affect in mania? *Psychopharmacology*, *231*(13), 2567–2577.

Perrier, E., Pompei, F., Ruberto, G., Vassos, E., Collier, D., & Frangou, S. (2011). Initial evidence for the role of *CACNA1C* on subcortical brain morphology in patients with bipolar disorder. *European Psychiatry*, *26*(3), 135–137.

Phelps, E. A., & LeDoux, J. E. (2005). Contributions of the Amygdala to Emotion Processing: From Animal Models to Human Behavior. *Neuron*, *48*(2), 175–187.

Pierce, R. C., & Kumaresan, V. (2006). The mesolimbic dopamine system: The final common pathway for the reinforcing effect of drugs of abuse? *Neuroscience & Biobehavioral Reviews*, *30*(2), 215–238. https://doi.org/10.1016/J.NEUBIOREV.2005.04.016

Pletnikov, M. V., Ayhan, Y., Nikolskaia, O., Xu, Y., Ovanesov, M. V., Huang, H., … Ross, C. A.

(2008). Inducible expression of mutant human DISC1 in mice is associated with brain and behavioral abnormalities reminiscent of schizophrenia. *Molecular Psychiatry*, *13*(2), 173–186. https://doi.org/10.1038/sj.mp.4002079

Poole, T. B., & Fish, J. (1976). An investigation of individual, age and sexual differences in the play of *Rattus norvegicus* (Mammalia*: Rodentia*). *Journal of Zoology*, *179*(2), 249–259.

Porsolt, R. D., Bertin, A., & Jalfre, M. (1977). Behavioral despair in mice: a primary screening test for antidepressants. *Archives Internationales de Pharmacodynamie et de Therapie*, *229*(2), 327–336. http://europepmc.org/abstract/MED/596982

Portavella, M., Depaulis, A., & Vergnes, M. (1993). 22–28 Khz ultrasonic vocalizations associated with defensive reactions in male rats do not result from fear or aversion. *Psychopharmacology*, *111*(2), 190–194. https://doi.org/10.1007/BF02245522

Portfors, C. V. (2007). Types and functions of ultrasonic vocalizations in laboratory rats and mice. *Journal of the American Association for Laboratory Animal Science*, *46*(1), 28–34.

Price, J. L., & Drevets, W. C. (2010). Neurocircuitry of Mood Disorders. *Neuropsychopharmacology*, *35*(1), 192–216.

Prickaerts, J., Moechars, D., Cryns, K., Lenaerts, I., van Craenendonck, H., Goris, I., … Steckler, T. (2006). Transgenic Mice Overexpressing Glycogen Synthase Kinase 3β: A Putative Model of Hyperactivity and Mania. *The Journal of Neuroscience*, *26*(35), 9022-9029. http://www.jneurosci.org/content/26/35/9022.abstract

Proulx, C. D., Hikosaka, O., & Malinow, R. (2014). Reward processing by the lateral habenula in normal and depressive behaviors. *Nature Neuroscience*, *17*(9), 1146–1152.

Pym, E., Sasidharan, N., Thompson-Peer, K. L., Simon, D. J., Anselmo, A., Sadreyev, R. I., … Kaplan, J. M. (2017). Shank is a dose-dependent regulator of Cav1 calcium current and CREB target expression. *eLife*, *6*, e18931.

Radua, J., Surguladze, S. A., Marshall, N., Walshe, M., Bramon, E., Collier, D. A., … McDonald, C. (2013). The impact of *CACNA1C* allelic variation on effective connectivity during emotional processing in bipolar disorder. *Molecular Psychiatry*, *18*(5), 526.

Rajadhyaksha, A., Husson, I., Satpute, S. S., Ku, K. D., Ren, J. Q., Guerriero, R. M., … Kosofsky, B. E. (2004). L-Type Ca 2+ Channels Mediate Adaptation of Extracellular in the Ventral Tegmental Area after Chronic Amphetamine Treatment. *The Journal of Neuroscience*, *24*(34), 7464–7476. https://doi.org/10.1523/JNEUROSCI.0612-04.2004

Ray, R. D., & Zald, D. H. (2012). Anatomical insights into the interaction of emotion and cognition in the prefrontal cortex. *Neuroscience & Biobehavioral Reviews*, *36*(1), 479–501.

Raza, S., Himmler, B. T., Himmler, S. M., Harker, A., Kolb, B., Pellis, S. M., & Gibb, R. (2015). Effects of prenatal exposure to valproic acid on the development of juvenile-typical social play in rats. *Behavioural Pharmacology*, *26*(8), 707–719.

Richards, D. (2011). Prevalence and clinical course of depression: A review. *Clinical Psychology*

Review, 31(7), 1117-1125. https://doi.org/10.1016/j.cpr.2011.07.004

Riga, D., Matos, M. R., Glas, A., Smit, A. B., Spijker, S., & Van den Oever, M. C. (2014). Optogenetic dissection of medial prefrontal cortex circuitry. *Frontiers in Systems Neuroscience*, *8*. https://doi.org/10.3389/fnsys.2014.00230

Ripke, S., Neale, B. M., Corvin, A., Walters, J. T. R., Farh, K.-H., Holmans, P. a., … O'Donovan, M. C. (2014). Biological insights from 108 schizophrenia-associated genetic loci. *Nature, 511*, 421-427. https://doi.org/10.1038/nature13595

Rippberger, H., van Gaalen, M. M., Schwarting, R. K. W., & Wohr, M. (2015). Environmental and Pharmacological Modulation of Amphetamine- Induced 50-kHz Ultrasonic Vocalizations in Rats. *Current Neuropharmacology*, *13*(2), 220–232.

Rolstad, S., Sellgren Majkowitz, C., Joas, E., Ekman, C. J., Pålsson, E., & Landén, M. (2016). Polymorphisms of BDNF and *CACNA1C* are not associated with cognitive functioning in bipolar disorder or healthy controls. *Cognitive Neuropsychiatry*, *21*(3), 271–278. https://doi.org/10.1080/13546805.2016.1185405

Rosenberg, R. E., Law, J. K., Yenokyan, G., McGready, J., Kaufmann, W. E., & Law, P. A. (2009). Characteristics and concordance of autism spectrum disorders among 277 twin pairs. *Archives of Pediatrics and Adolescent Medicine*, *163*(10), 907–914.

Roussos, P., Giakoumaki, S. G., Georgakopoulos, A., Robakis, N. K., & Bitsios, P. (2011). The *CACNA1C* and *ANK3* risk alleles impact on affective personality traits and startle reactivity but not on cognition or gating in healthy males. *Bipolar Disorders*, *13*(3), 250–259. https://doi.org/10.1111/j.1399-5618.2011.00924.x

Roussos, P., Mitchell, A. C., Voloudakis, G., Fullard, J. F., Pothula, V. M., Tsang, J., … Sklar, P. (2014). A Role for Noncoding Variation in Schizophrenia. *Cell Reports*, *9*(4), 1417–1429. https://doi.org/10.1016/j.celrep.2014.10.015

Russo, S. J., & Nestler, E. J. (2013). The Brain Reward Circuitry in Mood Disorders. *Nature Reviews. Neuroscience*, *14*(9), 609-625. https://doi.org/10.1038/nrn3381

Sadananda, M., Wöhr, M., & Schwarting, R. K. W. (2008). Playback of 22-kHz and 50-kHz ultrasonic vocalizations induces differential c-fos expression in rat brain. *Neuroscience Letters*, *435*(1), 17–23. https://doi.org/10.1016/J.NEULET.2008.02.002

Sahakian, B. J., Morris, R. G., Evenden, J. L., Heald, A., Levy, R., Philpot, M., & Robbins, T. W. (1988). A Comparative Study of Visospatial Memory and Learning in Alzheimer-Type Dementia and Parkinson's Disease. *Brain*, *111*(3), 695–718.

Salama, G., & London, B. (2007). Mouse models of long QT syndrome. *The Journal of Physiology*, *578*(1), 43–53. https://doi.org/10.1113/jphysiol.2006.118745

Sales, G. D. (1972). Ultrasound and aggressive behaviour in rats and other small mammals. *Animal Behaviour*, *20*(1), 88–100. https://doi.org/10.1016/S0003-3472(72)80177-5

Sanchez-Moreno, J., Martinez-Aran, A., Tabarés-Seisdedos, R., Torrent, C., Vieta, E., & Ayuso-Mateos, J. L. (2009). Functioning and Disability in Bipolar Disorder: An Extensive

Review. *Psychotherapy and Psychosomatics*, *78*(5), 285–297.

Santarelli, L. (2003). Requirement of Hippocampal Neurogenesis for the Behavioral Effects of Antidepressants. *Science*, *301*(5634), 805–809. https://doi.org/10.1126/science.1083328

Savla, G. N., Vella, L., Armstrong, C. C., Penn, D. L., & Twamley, E. W. (2013). Deficits in Domains of Social Cognition in Schizophrenia: A Meta-Analysis of the Empirical Evidence. *Schizophrenia Bulletin*, *39*(5), 979–992. https://doi.org/10.1093/schbul/sbs080

Schizophrenia Psychiatric Genome-Wide Association Study (GWAS) Consortium. (2011). Genome-wide association study identifies five new schizophrenia loci. *Nature Genetics, 43*, 969-976. https://doi.org/10.1038/ng.940

Schneider, M., & Koch, M. (2005). Deficient Social and Play Behavior in Juvenile and Adult Rats after Neonatal Cortical Lesion: Effects of Chronic Pubertal Cannabinoid Treatment. *Neuropsychopharmacology*, *30*(5), 944–957. https://doi.org/10.1038/sj.npp.1300634

Schwarting, R. K. W., Jegan, N., & Wöhr, M. (2007). Situational factors, conditions and individual variables which can determine ultrasonic vocalizations in male adult Wistar rats. *Behavioural Brain Research*, *182*(2), 208–222.

Schwarting, R. K. W., & Wöhr, M. (2012). On the relationships between ultrasonic calling and anxiety-related behavior in rats. *Brazilian Journal of Medical and Biological Research*, *45*(4), 337–348. https://doi.org/10.1590/S0100-879X2012007500038

Scott, J. P., & Marston, M. (1950). Critical Periods Affecting the Development of Normal and Mal-Adjustive Social Behavior of Puppies. *The Pedagogical Seminary and Journal of Genetic Psychology*, *77*(1), 25–60.

Seffer, D., Rippberger, H., Schwarting, R. K. W., & Wöhr, M. (2015). Pro-social 50-kHz ultrasonic communication in rats: post-weaning but not post-adolescent social isolation leads to social impairments—phenotypic rescue by re-socialization. *Frontiers in Behavioral Neuroscience*, *9*, 102. https://doi.org/10.3389/fnbeh.2015.00102

Sewell, G. D. (1967). Ultrasound in Adult Rodents. *Nature*, *215*(5100), 512–512. https://doi.org/10.1038/215512a0

Sewell, G. D. (2009). Ultrasound and mating behaviour in rodents with some observations on other behavioural situations. *Journal of Zoology*, *168*(2), 149–164.

Shao, Y., Guan, Y., Wang, L., Qiu, Z., Liu, M., Chen, Y., … Li, D. (2014). CRISPR/Cas-mediated genome editing in the rat via direct injection of one-cell embryos. *Nature Protocols*, *9*(10), 2493–2512. https://doi.org/10.1038/nprot.2014.171

Silverman, J. L., Yang, M., Lord, C., & Crawley, J. N. (2010). Behavioural phenotyping assays for mouse models of autism. *Nature Reviews Neuroscience*, *11*(7), 490–502.

Sinnegger-Brauns, M. J., Huber, I. G., Koschak, A., Wild, C., Obermair, G. J., Einzinger, U., … Striessnig, J. (2009). Expression and 1,4-dihydropyridine-binding properties of brain L-type calcium channel isoforms. *Molecular Pharmacology*, *75*(2), 407–414.

Sklar, P., Ripke, S., Scott, L. J., Andreassen, O. A., Cichon, S., Craddock, N., … Purcell, S. M.

(2011). Large-scale genome-wide association analysis of bipolar disorder identifies a new susceptibility locus near ODZ4. *Nature Genetics*, *43*(10), 977–983.

Sklar, P., Smoller, J. W., Fan, J., Ferreira, M. A., Perlis, R. H., Chambert, K., … Purcell, S. M. (2008). Whole-genome association study of bipolar disorder. *Molecular Psychiatry*, *13*(6), 558–569. https://doi.org/10.1038/sj.mp.4002151

Smith, P. K., & Lewis, K. (1985). Rough-and-tumble play, fighting, and chasing in nursery school children. *Ethology and Sociobiology*, *6*(3), 175–181.

Smoller, J. W., & Finn, C. T. (2003). Family, twin, and adoption studies of bipolar disorder. *American Journal of Medical Genetics*, *123C*(1), 48–58.

Smotherman, W. P., Bell, R. W., Starzec, J., Elias, J., & Zachman, T. A. (1974). Maternal responses to infant vocalizations and olfactory cues in rats and mice. *Behavioral Biology*, *12*(1), 55–66. https://doi.org/10.1016/S0091-6773(74)91026-8

Snoeren, E. M. S., & Ågmo, A. (2013). Female ultrasonic vocalizations have no incentive value for male rats. *Behavioral Neuroscience*. https://doi.org/10.1037/a0032027

Soeiro-de-Souza, M. G., Bio, D. S., Dias, V. V., Vieta, E., Machado-Vieira, R., & Moreno, R. A. (2013). The *CACNA1C* risk allele selectively impacts on executive function in bipolar type I disorder. *Acta Psychiatrica Scandinavica*. https://doi.org/10.1111/acps.12073

Sombers, L. A., Beyene, M., Carelli, R. M., & Wightman, R. M. (2009). Synaptic Overflow of Dopamine in the Nucleus Accumbens Arises from Neuronal Activity in the Ventral Tegmental Area. *Journal of Neuroscience*, *29*(6), 1735–1742.

Spanagel, R., & Weiss, F. (1999). The dopamine hypothesis of reward: past and current status. *Trends in Neurosciences*, *22*(11), 521–527.

Splawski, I., Timothy, K. W., Decher, N., Kumar, P., Sachse, F. B., Beggs, A. H., … Keating, M. T. (2005). Severe arrhythmia disorder caused by cardiac L-type calcium channel mutations. *Proceedings of the National Academy of Sciences, 10*(23), 8089-8096.

Splawski, I., Timothy, K. W., Sharpe, L. M., Decher, N., Kumar, P., Bloise, R., … Keating, M. T. (2004). $Ca_V1.2$ Calcium Channel Dysfunction Causes a Multisystem Disorder Including Arrhythmia and Autism. *Cell*, *119*(1), 19–31.

Squire, L. R., Stark, C. E. L., & Clark, R. E. (2004). The Medial Temporal Lobe. *Annual Review of Neuroscience*, *27*(1), 279–306.

Stockman, S. L., & McCarthy, M. M. (2017). Predator odor exposure of rat pups has opposite effects on play by juvenile males and females. *Pharmacology Biochemistry and Behavior*, *152*, 20–29.

Strohmaier, J., Amelang, M., Hothorn, L. A., Witt, S. H., Nieratschker, V., Gerhard, D., … Schulze, T. G. (2013). The psychiatric vulnerability gene *CACNA1C* and its sex-specific relationship with personality traits, resilience factors and depressive symptoms in the general population. *Molecular Psychiatry*, *18*(5), 607–613.

Sullivan, P. F., Neale, M. C., & Kendler, K. S. (2000). Genetic Epidemiology of Major

Depression: Review and Meta-Analysis. *American Journal of Psychiatry*, *157*(10), 1552–1562. https://doi.org/10.1176/appi.ajp.157.10.1552

Sumner, J. A., Sheridan, M. A., Drury, S. S., Esteves, K. C., Walsh, K., Koenen, K. C., & McLaughlin, K. A. (2015). Variation in *CACNA1C* is Associated with Amygdala Structure and Function in Adolescents. *Journal of Child and Adolescent Psychopharmacology*, *25*(9), 701–710. https://doi.org/10.1089/cap.2015.0047

Sungur, A. Ö., Schwarting, R. K. W., & Wöhr, M. (2017). Behavioral phenotypes and neurobiological mechanisms in the Shank1 mouse model for autism spectrum disorder: A translational perspective. *Behavioural Brain Research*, (in press).

Swainson, R., Hodges, J. R., Galton, C. J., Semple, J., Michael, A., Dunn, B. D., … Sahakian, B. J. (2001). Early Detection and Differential Diagnosis of Alzheimer's Disease and Depression with Neuropsychological Tasks. *Dementia and Geriatric Cognitive Disorders*, *12*(4), 265–280. https://doi.org/10.1159/000051269

Takahashi, L. K., Thomas, D. A., & Barfield, R. J. (1983). Analysis of Ultrasonic Vocalizations Emitted by Intruders During Aggressive Encounters Among Rats (*Rattus norvegicus)*. *Journal of Comparative Psychology*, 97(3), 201–206.

Tamborski H. A., & Hennessy, M. B. (1995). Corticotropin-releasing factor modulation of the ultrasonic vocalization rate of isolated rat pups. *Developmental Brain Research*, *87*(2), 125–134. https://doi.org/10.1016/0165-3806(95)00064-K

Taracha, E., Kaniuga, E., Wyszogrodzka, E., Płaźnik, A., Stefański, R., & Chrapusta, S. J. (2016). Poor sensitization of 50-kHz vocalization response to amphetamine predicts rat susceptibility to self-administration of the drug. *Psychopharmacology, 3*(14), 2827-2840. https://doi.org/10.1007/s00213-016-4328-4

Taylor, G. T. (1980). Fighting in juvenile rats and the ontogeny of agonistic behavior. *Journal of Comparative and Physiological Psychology, 94*(5), 953-961.

Taylor Tavares, J. V., Clark, L., Furey, M. L., Williams, G. B., Sahakian, B. J., & Drevets, W. C. (2008). Neural basis of abnormal response to negative feedback in unmedicated mood disorders. *NeuroImage*, *42*(3), 1118–1126.

Taylor, P. V., Veenema, A. H., Paul, M. J., Bredewold, R., Isaacs, S., & de Vries, G. J. (2012). Sexually dimorphic effects of a prenatal immune challenge on social play and vasopressin expression in juvenile rats. *Biology of Sex Differences*, *3*(1), 15.

Temme, S. J., Bell, R. Z., Fisher, G. L., & Murphy, G. G. (2016). Deletion of the Mouse Homolog of *CACNA1C* Disrupts Discrete Forms of Hippocampal-Dependent Memory and Neurogenesis within the Dentate Gyrus. *eNeuro*, *3*(6).

Terrillion, C. E., Dao, D. T., Cachope, R., Lobo, M. K., Puche, A. C., Cheer, J. F., & Gould, T. D. (2017). Reduced levels of *CACNA1C* attenuate mesolimbic dopamine system function. *Genes, Brain and Behavior*, *16*(5), 495–505. https://doi.org/10.1111/gbb.12371

Terrillion, C. E., Francis, T. C., Puche, A. C., Lobo, M. K., & Gould, T. D. (2017). Decreased Nucleus Accumbens Expression of Psychiatric Disorder Risk Gene *CACNA1C* Promotes

Susceptibility to Social Stress. *International Journal of Neuropsychopharmacology*, *20*(5), 428–433. https://doi.org/10.1093/ijnp/pyw112

Tesli, M., Skatun, K. C., Ousdal, O. T., Brown, A. A., Thoresen, C., Agartz, I., … Andreassen, O. A. (2013). *CACNA1C* Risk Variant and Amygdala Activity in Bipolar Disorder, Schizophrenia and Healthy Controls. *PLOS ONE*, *8*(2), e56970.

Thimm, M., Kircher, T., Kellermann, T., Markov, V., Krach, S., Jansen, A., … Krug, A. (2010). Effects of a *CACNA1C* genotype on attention networks in healthy individuals. *Psychological Medicine, 41*(7), 1551-1561. https://doi.org/10.1017/S0033291710002217

Thomas, D. A., Howard, S. B., & Barfield, R. J. (1982). Male-produced ultrasonic vocalizations and mating patterns in female rats. *Journal of Comparative Psychology, 96*(5), 807-815. https://doi.org/10.1037/h0077919

Thomas, D. A., Talalas, L., & Barfield, R. J. (1981). Effect of devocalization of the male on mating behavior in rats. *Journal of Comparative and Physiological Psychology, 95*(4), 630-637. https://doi.org/10.1037/h0077803

Thompson, B., Leonard, K. C., & Brudzynski, S. M. (2006). Amphetamine-induced 50 kHz calls from rat nucleus accumbens: A quantitative mapping study and acoustic analysis. *Behavioural Brain Research*, *168*(1), 64–73. https://doi.org/10.1016/j.bbr.2005.10.012

Thor, D. H., & Holloway, W. R. (1986). Social play soliciting by male and female juvenile rats: Effects of neonatal androgenization and sex of cagemates. *Behavioral Neuroscience*, *100*(2), 275–279. https://doi.org/10.1037/0735-7044.100.2.275

Tong, C., Huang, G., Ashton, C., Li, P., & Ying, Q.-L. (2011). Generating gene knockout rats by homologous recombination in embryonic stem cells. *Nature Protocols*, *6*(6), 827–844. https://doi.org/10.1038/nprot.2011.338

Trezza, V., Baarendse, P. J. J., & Vanderschuren, L. J. (2010). The pleasures of play: pharmacological insights into social reward mechanisms. *Trends in Pharmacological Sciences*, *31*(10), 463–469. https://doi.org/10.1016/j.tips.2010.06.008

Trezza, V., Baarendse, P. J., & Vanderschuren, L. J. (2009). Prosocial Effects of Nicotine and Ethanol in Adolescent Rats Through Partially Dissociable Neurobehavioral Mechanisms. *Neuropsychopharmacology*, *34*(12), 2560–2573. https://doi.org/10.1038/npp.2009.85

Trezza, V., & Vanderschuren, L. J. (2008). Bidirectional cannabinoid modulation of social behavior in adolescent rats. *Psychopharmacology*, *197*(2), 217–227.

Tsai, S.-Y., Lee, H.-C., Chen, C.-C., & Huang, Y.-L. (2007). Cognitive impairment in later life in patients with early-onset bipolar disorder. *Bipolar Disorders*, *9*(8), 868–875. https://doi.org/10.1111/j.1399-5618.2007.00498.x

Van Den Berg, C. L., Hol, T., Van Ree, J. M., Spruijt, B. M., Everts, H., & Koolhaas, J. M. (1999). Play is indispensable for an adequate development of coping with social challenges in the rat. *Developmental Psychobiology*, *34*(2), 129–138.

Van Den Berg, C. L., van Ree, J. M., & Spruijt, B. M. (1999). Play deprivation decreases adult

social behavior. *Rudolf Magnus Institute for Neuroscience, Utrecht University*.

Van Der Poel, A. M., & Miczek, K. A. (1991). Long Ultrasonic Calls in Male Rats Following Mating, Defeat and Aversive Stimulation: Frequency Modulation and Bout Structure. *Behaviour*, *119*(1), 127–142. https://doi.org/10.1163/156853991X00409

van der Staay, F. J., Arndt, S. S., & Nordquist, R. E. (2009). Evaluation of animal models of neurobehavioral disorders. *Behavioral and Brain Functions*, *5*(1), 11.

van Gorp, W. G., Altshuler, L., Theberge, D. C., & Mintz, J. (1999). Declarative and procedural memory in bipolar disorder. *Biological Psychiatry*, *46*(4), 525–531.

van Kerkhof, L. W. M., Damsteegt, R., Trezza, V., Voorn, P., & Vanderschuren, L. J. (2013). Functional integrity of the habenula is necessary for social play behaviour in rats. *European Journal of Neuroscience*, *38*(10), 3465–3475.

van Kerkhof, L. W. M., Trezza, V., Mulder, T., Gao, P., Voorn, P., & Vanderschuren, L. J. (2013). Cellular activation in limbic brain systems during social play behaviour in rats. *Brain Structure and Function, 219*(4), 1181-1211. https://doi.org/10.1007/s00429-013-0558-y

Vanderschuren, L. J., Achterberg, E. J. M., & Trezza, V. (2016). The neurobiology of social play and its rewarding value in rats. *Neuroscience and Biobehavioral Reviews*, *70*, 86–105.

Vanderschuren, L. J., Niesink, R. J., & Van Ree, J. M. (1997). The neurobiology of social play behavior in rats. *Neuroscience and Biobehavioral Reviews*, *21*(3), 309–326. https://doi.org/10.1016/S0149-7634(96)00020-6

Vanderschuren, L. J., Stein, E. A., Wiegant, V. M., & Van Ree, J. M. (1995). Social isolation and social interaction alter regional brain opioid receptor binding in rats. *European Neuropsychopharmacology*, *5*(2), 119–127.

Vanderschuren, L. J., & Trezza, V. (2013). What the Laboratory Rat has Taught us About Social Play Behavior: Role in Behavioral Development and Neural Mechanisms. In *The Neurobiology of Childhood* (pp. 189–212). Springer Berlin Heidelberg

Vanderschuren, L. J., Trezza, V., Griffioen-Roose, S., Schiepers, O. J. G., Van Leeuwen, N., De Vries, T. J., & Schoffelmeer, A. N. M. (2008). Methylphenidate Disrupts Social Play Behavior in Adolescent Rats. *Neuropsychopharmacology*, *33*(12), 2946–2956.

Wallace, T. L., Ballard, T. M., Pouzet, B., Riedel, W. J., & Wettstein, J. G. (2011). Drug targets for cognitive enhancement in neuropsychiatric disorders. *Pharmacology Biochemistry and Behavior*, *99*(2), 130–145. https://doi.org/10.1016/J.PBB.2011.03.022

Wang, F., Mcintosh, A. M., He, Y., Gelernter, J., & Blumberg, H. P. (2011). The association of genetic variation in *CACNA1C* with structure and function of a frontotemporal system. *Bipolar Disorders*, *13*(7–8), 696–700. https://doi.org/10.1111/j.1399-5618.2011.00963.x

Ward, I. L., & Stehm, K. E. (1991). Prenatal stress feminizes juvenile play patterns in male rats. *Physiology & Behavior*, *50*(3), 601–605.

Watson, D. M., & Croft, D. B. (1993). Playfighting in Captive Red-Necked Wallabies, Macropus Rufogriseus Banksianus. *Behaviour*, *126*(3), 219–245.

Webber, E. S., Harmon, K. M., Beckwith, T. J., Peña, S., Burgdorf, J., Panksepp, J., & Cromwell, H. C. (2012). Selective breeding for 50kHz ultrasonic vocalization emission produces alterations in the ontogeny and regulation of rough-and-tumble play. *Behavioural Brain Research*, *229*(1), 138–144. https://doi.org/10.1016/j.bbr.2012.01.012

Weiss, S. M., Lightowler, S., Stanhope, K. J., Kennett, G. A., & Dourish, C. T. (2000). Measurement of anxiety in transgenic mice. *Reviews in the Neurosciences*, *11*(1), 59–74. http://www.ncbi.nlm.nih.gov/pubmed/10716656

Wellmann, K. A., George, F., Brnouti, F., & Mooney, S. M. (2015). Docosahexaenoic acid partially ameliorates deficits in social behavior and ultrasonic vocalizations caused by prenatal ethanol exposure. *Behavioural Brain Research*, *286*, 201–211.

Wendler, E., de Souza, C. P., Vecchia, D. D., Kanazawa, L. K. S., de Almeida Soares Hocayen, P., Wöhr, M., … Andreatini, R. (2016). Evaluation of 50-kHz ultrasonic vocalizations in animal models of mania: Ketamine and lisdexamfetamine-induced hyperlocomotion in rats. *European Neuropsychopharmacology*, *26*(12), 1900–1908.

Wessa, M., Linke, J., Witt, S. H., Nieratschker, V., Esslinger, C., Kirsch, P., … Rietschel, M. (2010). The *CACNA1C* risk variant for bipolar disorder influences limbic activity. *Molecular Psychiatry, 15*, 1126-1127. https://doi.org/10.1038/mp.2009.103

Whishaw, I. Q., & Kolb, B. (2009). *The Behavior of the Laboratory Rat: A Handbook with Tests*. *The Behavior of the Laboratory Rat: A Handbook with Tests*. Oxford University Press. https://doi.org/10.1093/acprof:oso/9780195162851.001.0001

White, J. A., McKinney, B. C., John, M. C., Powers, P. A., Kamp, T. J., & Murphy, G. G. (2008). Conditional forebrain deletion of the L-type calcium channel CaV1.2 disrupts remote spatial memories in mice. *Learning & Memory*, *15*(1), 1–5.

White, N. R., & Barfield, R. J. (1987). Role of the ultrasonic vocalization of the female rat (*Rattus norvegicus*) in sexual behavior. *Journal of Comparative Psychology*, *101*(1), 73–81. https://doi.org/10.1037/0735-7036.101.1.73

Willadsen, M., Seffer, D., Schwarting, R. K. W., & Wöhr, M. (2014). Rodent ultrasonic communication: Male prosocial 50-kHz ultrasonic vocalizations elicit social approach behavior in female rats (Rattus norvegicus). *Journal of Comparative Psychology*, *128*(1), 56–64. https://doi.org/10.1037/a0034778

Williams, S. N., & Undieh, A. S. (2010). Brain-Derived Neurotrophic Factor Signaling Modulates Cocaine Induction of Reward-Associated Ultrasonic Vocalization in Rats. *Journal of Pharmacology and Experimental Therapeutics*, *332*(2), 463–468.

Willuhn, I., Tose, A., Wanat, M. J., Hart, A. S., Hollon, N. G., Phillips, P. E. M., … Wöhr, M. (2014). Phasic Dopamine Release in the Nucleus Accumbens in Response to Pro-Social 50 kHz Ultrasonic Vocalizations in Rats. *The Journal of Neuroscience*, *34*(32), 10616-10623. https://doi.org/10.1523/JNEUROSCI.1060-14.2014

Wilson, C. A., & Terry, A. V. (2013). Variable maternal stress in rats alters locomotor activity, social behavior, and recognition memory in the adult offspring. *Pharmacology*

Biochemistry and Behavior, *104*, 47–61. https://doi.org/10.1016/J.PBB.2012.12.015

Wöhr, M. (2018). Ultrasonic communication in rats: appetitive 50-kHz ultrasonic vocalizations as social contact calls. *Behavioral Ecology and Sociobiology*, *72*(1), 14.

Wöhr, M., Borta, A., & Schwarting, R. K. W. (2005). Overt behavior and ultrasonic vocalization in a fear conditioning paradigm: A dose-response study in the rat. *Neurobiology of Learning and Memory*, *84*(3), 228–240.

Wöhr, M., Engelhardt, K. A., Seffer, D., Sungur, A. Ö., & Schwarting, R. K. W. (2015). Acoustic Communication in Rats: Effects of Social Experiences on Ultrasonic Vocalizations as Socio-affective Signals. In M. Wöhr & S. Krach (Eds.), *Social Behavior from Rodents to Humans: Neural Foundations and Clinical Implications* (pp. 67–89). Switzerland: Springer International Publishing.

Wöhr, M., Houx, B., Schwarting, R. K. W., & Spruijt, B. (2008). Effects of experience and context on 50-kHz vocalizations in rats. *Physiology and Behavior*, *93*(4–5), 766–776.

Wöhr, M., & Scattoni, M. L. (2013). Behavioural methods used in rodent models of autism spectrum disorders: Current standards and new developments. *Behavioural Brain Research*, *251*, 5–17. https://doi.org/10.1016/j.bbr.2013.05.047

Wöhr, M., & Schwarting, R. K. W. (2007). Ultrasonic communication in rats: Can playback of 50-kHz calls induce approach behavior? *PLoS ONE*, *2*(12), e1365.

Wöhr, M., & Schwarting, R. K. W. (2008a). Maternal care, isolation-induced infant ultrasonic calling, and their relations to adult anxiety-related behavior in the rat. *Behavioral Neuroscience*, *122*(2), 310–330. https://doi.org/10.1037/0735-7044.122.2.310

Wöhr, M., & Schwarting, R. K. W. (2008b). Ultrasonic calling during fear conditioning in the rat: no evidence for an audience effect. *Animal Behaviour*, *76*(3), 749–760.

Wöhr, M., & Schwarting, R. K. W. (2009). Ultrasonic communication in rats: Effects of morphine and naloxone on vocal and behavioral responses to playback of 50-kHz vocalizations. *Pharmacology Biochemistry and Behavior*, *94*(2), 285–295.

Wöhr, M., & Schwarting, R. K. W. (2013). Affective communication in rodents: Ultrasonic vocalizations as a tool for research on emotion and motivation. *Cell and Tissue Research, 354*(1), 81-97. https://doi.org/10.1007/s00441-013-1607-9

Wöhr, M., van Gaalen, M. M., & Schwarting, R. K. W. (2015). Affective communication in rodents. *Behavioural Pharmacology*, *26*(6), 506–521.

World Health Organization. (1992). *The ICD-10 classification of metnal and behavioural disorders: Clinical and diagnostic guidelines* (6th ed.). Geneva: World Health Organization.

World Health Organization. Autism spectrum disorders. (2017, April 04). Autism spectrum disorders. Retrieved from http://www.who.int/en/news-room/fact-sheets/detail/autism-spectrum-disorders

World Health Organization. Depression. (2018, March 22). Depression. Retrieved from

http://www.who.int/en/news-room/fact-sheets/detail/depression

World Health Organization. Mental Disorders. (2017, April 28). Retrieved from http://www.who.int/en/news-room/fact-sheets/detail/mental-disorders

Wray, N. R., Pergadia, M. L., Blackwood, D. H. R., Penninx, B. W. J. H., Gordon, S. D., Nyholt, D. R., … Sullivan, P. F. (2012). Genome-wide association study of major depressive disorder: new results, meta-analysis, and lessons learned. *Molecular Psychiatry*, 17, 36-48. https://doi.org/10.1038/mp.2010.109

Wright, J. M., Dobosiewicz, M. R. S., & Clarke, P. B. S. (2012). α- and β-Adrenergic Receptors Differentially Modulate the Emission of Spontaneous and Amphetamine-Induced 50-kHz Ultrasonic Vocalizations in Adult Rats. *Neuropsychopharmacology*, *37*(3), 808–821. https://doi.org/10.1038/npp.2011.258

Wright, J. M., Gourdon, J. C., & Clarke, P. B. S. (2010). Identification of multiple call categories within the rich repertoire of adult rat 50-kHz ultrasonic vocalizations: effects of amphetamine and social context. *Psychopharmacology*, *211*(1), 1–13.

Yoo, J., Bakes, J., Bradley, C., Collingridge, G. L., & Kaang, B.-K. (2014). Shank mutant mice as an animal model of autism. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *369*(1633), 20130143.

Yoshimizu, T., Pan, J. Q., Mungenast, a E., Madison, J. M., Su, S., Ketterman, J., … Tsai, L.-H. (2015). Functional implications of a psychiatric risk variant within *CACNA1C* in induced human neurons. *Molecular Psychiatry, 20,* 162-169.

Young, J. W., Henry, B. L., & Geyer, M. A. (2011). Predictive animal models of mania: hits, misses and future directions. *British Journal of Pharmacology*, *164*(4), 1263–1284. https://doi.org/10.1111/j.1476-5381.2011.01318.x

Young, J. W., Powell, S. B., Risbrough, V., Marston, H. M., & Geyer, M. A. (2009). Using the MATRICS to guide development of a preclinical cognitive test battery for research in schizophrenia. *Pharmacology & Therapeutics*, *122*(2), 150–202.

Zaccaroni, M., Massolo, A., Della Seta, D., Farabollini, F., Giannelli, G., Fusani, L., & Dessì-Fulgheri, F. (2017). Developmental Exposure to Low Levels of Ethinylestradiol Affects Play Behavior in Juvenile Female Rats. *Neurotoxicity Research, 33*(4), 876-886. https://doi.org/10.1007/s12640-017-9852-4

Zamponi, G. W. (2016). Targeting voltage-gated calcium channels in neurological and psychiatric diseases. *Nature Reviews. Drug Discovery*, *15*(1), 19–34.

Zan, Y., Haag, J. D., Chen, K.-S., Shepel, L. A., Wigington, D., Wang, Y.-R., … Gould, M. N. (2003). Production of knockout rats using ENU mutagenesis and a yeast-based screening assay. *Nature Biotechnology*, *21*(6), 645–651. https://doi.org/10.1038/nbt830

Zanos, P., Bhat, S., Terrillion, C. E., Smith, R. J., Tonelli, L. H., & Gould, T. D. (2015). Sex-dependent modulation of age-related cognitive decline by the L-type calcium channel gene *CACNA1C* ($Ca_v1.2$). *European Journal of Neuroscience*, *42*(8), 2499–2507.

Zhang, Q., Shen, Q., Xu, Z., Chen, M., Cheng, L., Zhai, J., … Chen, C. (2011). The Effects of *CACNA1C* Gene Polymorphism on Spatial Working Memory in Both Healthy Controls and Patients with Schizophrenia or Bipolar Disorder. *Neuropsychopharmacology*, *37*(10), 677-684. https://doi.org/10.1038/npp.2011.242

Zhang, X.-F., Cooper, D. C., & White, F. J. (2002). Repeated Cocaine Treatment Decreases Whole-Cell Calcium Current in Rat Nucleus Accumbens Neurons. *Journal of Pharmacology and Experimental Therapeutics*, *301*(3), 1119–1125.

Zuccotti, A., Clementi, S., Reinbothe, T., Torrente, A., Vandael, D. H., & Pirone, A. (2011). Structural and functional differences between L-type calcium channels: Crucial issues for future selective targeting. *Trends in Pharmacological Sciences, 32*(6), 366-375. https://doi.org/10.1016/j.tips.2011.02.012

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Erklärung

Hiermit versichere ich, dass ich die vorliegende Dissertation:

"Sex-Specific Effects of Cacna1c Haploinsufficiency on Social Behavior, Ultrasonic Communication, and Cognition in Rats"

selbstständig, ohne unerlaubte Hilfe angefertigt und mich dabei keiner anderen als der von mir ausdrücklich bezeichneten Quellen und Hilfen bedient habe.

Die Dissertation wurde in der jetzigen oder einer ähnlichen Form noch bei keiner anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

Marburg, 07.05.2018

Theresa Kisko

Theresa Marie Kisko