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Effects of *Cacna1c* Haploinsufficiency and Environmental Impact on Spatial Learning, Cognitive Flexibility and Social Behavior in Rats

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Abbreviations

ACC	anterior cingulate cortex
AMPH	amphetamine
ASD	autism spectrum disorder
BDNF	brain-derived neurotrophic factor
BPD	bipolar disorder
CNS	central nervous system
CUS	chronic unpredictable stress
DA	dopamine
DG	dentate gyrus
DSM	Diagnostic and Statistical Manual of Mental Disorders
ENR	enrichment
GWAS	genome-wide association study
GxE	gene x environment interaction
HC	hippocampus
HET	heterozygous
ISO	social isolation
ITI	inter-trial interval
LTCC	L-type voltage-gated calcium channels
LTP	long-term potentiation
MDD	major depressive disorder
MWM	Morris water maze
NAcc	Nucleus accumbens
NOR	novel object recognition
PFC	prefrontal cortex
PND	postnatal day
RAM	radial arm maze
RL	reversal learning
RM	reference memory
SCZ	schizophrenia
SL	spatial learning
SNP	single nucleotide polymorphism
TLE	threatening life event
TS	Timothy syndrome
USV	ultrasonic vocalization(s)
VTA	ventral tegmental area
WM	working memory
WT	wildtype

Summary

Mental disorders affect a great number of people worldwide and are highly debilitating. While genetic and environmental influences are thought to contribute to their etiology, the exact mechanisms are still poorly understood. The psychiatric risk gene *CACNA1C* codes for the α_{1c} subunit of voltage-gated calcium channels and has been associated with major depression, bipolar disorder, schizophrenia and autism spectrum disorders in genome-wide association studies and was further implicated by clinical studies and those using genetically altered mice. In an effort to elucidate how *CACNA1C* interacts with environmental influences to confer disorder risk, this dissertation used a newly developed constitutive *Cacna1c* heterozygous rat model to examine male and female animals in paradigms aimed at cognitive abilities and social behavior, both of which frequently found dysfunctional in neuropsychiatric disorders. In Study I and II, sex-specific effects of *Cacna1c* haploinsufficiency were discovered on rough-and-tumble play, emission of ultrasonic vocalizations (USV) and behavioral reactions in the USV playback paradigm, indicative of altered salience coding for social stimuli and reduced incentive value of pro-social interactions. For Study III, male and female rats were subjected to spatial learning and relearning on the radial arm maze, as well as to novel object recognition. The same paradigms were applied in Study IV, in which animals were previously exposed to four weeks of either post-weaning social isolation, standard housing or social and physical enrichment during the critical juvenile developmental period. Compared to the prominent social deficits in Study I and II, intact spatial and reversal learning abilities were seen in *Cacna1c* heterozygous animals with initial perseverative tendencies in males. Enrichment had an overall positive effect on learning and cognitive flexibility, whereas social isolation caused a profound impairment in object recognition, regardless of genotype. Furthermore, deficits observed in heterozygous animals under standard housing conditions were rescued by enriched rearing conditions, in the sense of a gene x environment interaction. Together, this dissertation adds to the growing body of evidence suggesting that a variation in *Cacna1c* genotype is causally involved in social and cognitive dysfunction as core phenotypes of various neuropsychiatric disorders, yet that individuals harboring genetic risk may benefit from early intervention and positive environmental influence.

Zusammenfassung

Psychische Erkrankungen betreffen weltweit eine große Anzahl von Menschen und führen zu außerordentlich negativen Konsequenzen. Es wird angenommen, dass sowohl genetische, als auch Umwelteinflüsse zu ihrer Entstehung beitragen, jedoch sind die genauen Mechanismen noch wenig bekannt. Das Risikogen *CACNA1C* kodiert die α_{1c} Untereinheit von spannungsgesteuerten Kalziumkanälen und wurde in mehreren genomweiten Assoziationsstudien, klinischen Studien und Experimenten an genetisch veränderten Mäusen mit Depressionen, bipolaren Störungen, Schizophrenie und Autismus-Spektrum-Störungen in Verbindung gebracht. Um herauszufinden wie die Interaktion von *CACNA1C* mit Umwelteinflüssen das Risiko für eine solche Erkrankung erhöht, bedient sich diese Dissertation eines neu entwickelten konstitutiven heterozygoten *Cacna1c*-Rattenmodells und untersucht die männlichen und weiblichen Tiere in Paradigmen, die auf soziales Verhalten und kognitive Fähigkeiten abzielen, welche bei neuropsychiatrischen Erkrankungen häufig als dysfunktional eingestuft werden. In den Studien I und II wurden geschlechtsspezifische Effekte der *Cacna1c*-Haploinsuffizienz auf das soziale Spielverhalten, die Produktion von Ultraschallvokalisationen (USV) und Verhaltensreaktionen im USV-Playback Paradigma aufgedeckt, was auf eine veränderte Salienz-Kodierung für soziale Reize und einen verringerten Anreizwert von prosozialen Interaktionen hinweist. Für Studie III wurden männliche und weibliche Ratten räumlichem Lernen und Umlernen im Radialarmlabyrinth sowie einem Paradigma zur Objektwiedererkennung unterzogen. Die gleichen Paradigmen wurden in Studie IV angewendet, in welcher die Tiere während der kritischen Entwicklungsphase nach dem Absetzen für vier Wochen entweder in sozialer Isolation, in Standardkäfigen oder in sozial und physisch angereicherten Umgebungen gehalten wurden. Im Vergleich zu den auffälligen sozialen Defiziten in Studie I und II wurden bei heterozygoten *Cacna1c*-Tieren neben einer Neigung zur Perseveration in männlichen Ratten allgemein intakte räumliche Lern- und Umlernfähigkeiten beobachtet. Die Umgebungsanreicherung wirkte sich insgesamt positiv auf das Lernen und die kognitive Flexibilität aus, während die soziale Isolation unabhängig vom Genotyp eine hochgradige Beeinträchtigung der Objekterkennung verursachte. Darüber hinaus wurden Defizite, die bei heterozygoten Tieren unter Standardhaltung beobachtet wurden, durch angereicherte Aufzuchtbedingungen im Sinne einer Gen x Umwelt Wechselwirkung behoben. Zusammengenommen ergänzt diese Dissertation die wachsende Zahl an Studien, die darauf hindeuten, dass eine Variation des *Cacna1c*-Genotyps kausal an sozialen und kognitiven Dysfunktionen als Kernphänotypen verschiedener neuropsychiatrischer Erkrankungen beteiligt ist, dass jedoch Personen mit genetischem Risiko von einer frühzeitigen Intervention und positiven Umwelteinflüssen profitieren können.

Mental disorders

Neuropsychiatric disorders, such as major depressive disorder (MDD), bipolar disorder (BPD), schizophrenia (SCZ), and autism spectrum disorder (ASD) affect a large number of people worldwide. As widespread, heritable, long-lasting, and highly debilitating illnesses, they present a source of great suffering for the affected individuals and their social environment, as well as a financial burden to society. However, while we already know that genetic, as well as environmental factors appear to contribute to their etiology, these pervasive afflictions remain poorly understood.

Prevalence

As expressed in a paper on the burden of mental illness, psychiatric diseases "affect most of the population in their lifetime" (Vigo et al., 2016, p. 176). Large-scale studies over the last two decades have estimated the lifetime prevalence of mood disorders at between 14% (Alonso et al., 2004) and just below 21% (Kessler et al., 2005), indicating that about one out of six people will suffer from major depression or bipolar disorder at least once. With a lifetime prevalence of 2-3% (Vieta et al., 2018), BPD occurs more seldom compared to MDD, although some authors believe bipolar type II, specifically, might still be underdiagnosed (Fagiolini et al., 2013).

Mood disorders seem to cluster in families, where the heritability of MDD is generally thought to be about 30-40% (Otte et al., 2016; Sullivan et al., 2000) with greater estimates in twin studies (Corfield et al., 2017). Looking at BPD, the level of heritability appears to be substantially higher, with up to 85% (McGuffin et al., 2003). Similar estimates have been made for SCZ and ASD. Both of these diseases are believed to be highly heritable at approximately 50-80% (Bai et al., 2019; Ivanov et al., 2015; Lai et al., 2014; Lichtenstein et al.; Sullivan et al., 2003), whereas their lifetime prevalence lies at just under 1% for SCZ (Kahn et al., 2015; McGrath et al., 2008) and around 1-2% for ASD worldwide (Harrington & Allen, 2014; Lai et al., 2014).

Once diagnosed, most of these disorders are characterized by their long-term course and high rates of recurrence. In the Munich 15-year follow-up study, an episodic-remitting course of affective disorders was reported for 90% of all diagnosed patients (Möller et al., 2011). Other authors found cumulative relapse rates of over 75% for depressive episodes in MDD patients at a 10 year follow-up, with treatment making little difference to this outcome (Conradi et al., 2017). BPD diagnoses appear to be of similar chronic quality (Fagiolini et al., 2013; Ferrari et al., 2016; Oswald et al., 2007). Comparable findings were also obtained by studies on SCZ, with 57-61% of patients displaying a chronic course over 15 years, dependent on the choice of diagnostic manual (Möller et al., 2011). Only a small percentage

of SCZ patients are reported to successfully enter the stage of recovery (Jääskeläinen et al., 2013) or even remission (Lambert et al., 2010).

Consequences

Ranging from the impact on society to the consequences the patients themselves have to face, the negative effects of mental disorders are manifold and severe. Numerous studies have investigated the economic and financial burden caused by mental illness. Throughout, affective disorders, SCZ and ASD cause a significant reduction in the likelihood of paid employment (Grande et al., 2013; Harvey, 2014; Howlin et al., 2013; Zwerling et al., 2002) at reported unemployment rates of up to 70% of diagnosed patients (Fajutrao et al., 2009). With a diagnosis of MDD or BPD, the increase in absenteeism from work is 50% higher compared to undiagnosed employees (Goldberg & Ernst, 2002; Simon, 2003) and when present, workers are reported to display a noticeable loss of productivity (Goldberg & Ernst, 2002). MDD and BPD patients further cause an increase in overall health service cost of 50-250% compared to other medical outpatients (Simon, 2003).

General social withdrawal, as a concomitant of diagnosis (Parker et al., 2018) is known to strongly impair patients' social relationships (Harvey, 2014). The family and caregiver burden is substantial (Perlick et al., 2001; Simon, 2003), due to a reduced capability to live independently (Billstedt & Gillberg, 2005; Harvey, 2014), difficulties regarding financial debts (Parker et al., 2018) as well as a serious increase in the risk for marital instability, and higher divorce and separation rates (Simon, 2003; Suppes et al., 2001).

Regarding the affected patients themselves, they experience decreased wellbeing (Parker et al., 2018; Simon, 2003), functional impairments (Morey-Nase et al., 2019), as well as lower life satisfaction in general (Parker et al., 2018). In those with ASD, perceived quality of life is reduced even in those without intellectual disability (Howlin et al., 2013). Individuals with a diagnosed neuropsychiatric disorder face internal and external stigma and negative stereotyping associated with mental illness (Gerlinger et al., 2013; Vigo et al., 2016), as well as an increased risk of developing mental and physical comorbidities such as anxiety, panic attacks, diabetes, strokes and heart disease (Kessler et al., 2005; Pané-Farré et al., 2013; Penninx et al., 2013). Over the last few years, global studies on the burden of disease have found MDD, BPD and SCZ to be among the leading causes for years of life lost to disability (GBD 2013 Mortality and Causes of Death Collaborators, 2015; GBD 2016 Disease and Injury Incidence and Prevalence Collaborators., 2017), with MDD scoring in the Top 10 causes in all but four of the examined countries in 2016 (GBD 2016 Disease and Injury Incidence and Prevalence Collaborators., 2017). On top of these non-fatal personal consequences, affected individuals have a highly increased mortality, with the likelihood of dying enlarged by 60-80% in MDD (Otte et al., 2016), and more than

100% in SCZ and ASD (McGrath et al., 2008; Woolfenden et al., 2012). With a diagnosis of SCZ, for example, the average life expectancy is reduced by approximately 20 years (Laursen et al., 2014). One major cause of death here is suicide, which is 13-20x more likely to be committed by patients of SCZ, MDD and BPD as compared to healthy controls of the same gender and economic status (Chesney et al., 2014; McGrath et al., 2008), with almost every second BPD patient attempting the act at least once in their lifetime (Gonda et al., 2012).

All of the listed consequences are exacerbated in patients suffering from comorbidities (Suppes et al., 2001), and some authors argue that even now, the weight of the global burden caused by mental disorders is still being underestimated (Vigo et al., 2016). In combination with the fact that most of these disorders affect a large percentage of people worldwide and usually follow a chronic course, it is all the more astounding that their etiology still remains poorly understood to date.

Etiology

It is widely considered true that MDD, BPD, SCZ and ASD are not Mendelian disorders – and thus not caused by one single genetic locus – but rather complex diseases, meaning they result from an interplay of multiple factors, such as genetic and environmental influences and their interaction (Uitterlinden, 2016). The heritability estimates for MDD, for example, suggest that only approximately one third of the disease risk can be explained by genetic components (Rao et al., 2016; Saveanu & Nemeroff, 2012). However, the elucidation of its genetic architecture is encumbered by the high symptom heterogeneity of MDD (Mullins & Lewis, 2017; van der Auwera et al., 2018), which is best characterized by a bias towards negative emotions, a diminished interest in almost all activities and psychomotor symptoms, but can also encompass impaired cognitive abilities and significant changes in body weight or sleep patterns (Hasler et al., 2004; Otte et al., 2016). Despite its high heritability, though, there are still no validated, biological tests that can be used for diagnosis and only a meager number of specific genes has been singled out so far (Mullins & Lewis, 2017; Saveanu & Nemeroff, 2012; van der Auwera et al., 2018). Once identified, most of the common risk genes for MDD only explain a small fraction of disease risk, which is why the current view on MDD etiology favors a gene x environment (GxE) interaction, meaning that a genetic vulnerability, for example, may lead to a diagnosis of MDD if certain environmental adversities come into play (Rao et al., 2016; Saveanu & Nemeroff, 2012). The fact remains that the biological basis of MDD is not well understood which hinders the development of new treatment methods. The same can be said for bipolar disorder.

Similar to major depression, BPD is a mood disorder with manifold symptoms, but with a clear distinction from MDD, according to the Diagnostic and Statistical Manual of Mental Disorders (DSM-5), by interloping episodes of mood swings between depressive episodes, ranging from hypomania to

mania or even psychosis, and often accompanied by a euphoric mood and a decreased need for sleep. Several family, twin and adoption studies have shown BPD to be one of the most heritable psychiatric disorders, mirrored by an increased risk with closer genetic relationship to a diagnosed patient (Craddock & Sklar, 2013). Here, too, no single genes with a strong impact on BPD have been identified. Instead, it is assumed that multiple genes with high prevalence in the general population each contribute a small individual share to an aggregated polygenic disease risk (Rowland & Marwaha, 2018) or that BPD results from a combination of common and rarer susceptibility variants (Sklar et al., 2011). Then again, twin studies suggest that it is not only genes that determine BPD, but that psychosocial and neurodevelopmental risk factors need to be taken into account (Craddock & Sklar, 2013; Marangoni et al., 2016).

The high heritability estimates from twin and adoption studies also point towards a strong genetic predisposition for SCZ (Cattane et al., 2018), a disorder that encompasses so called "positive" symptoms like hallucinations or delusions, "negative" symptoms such as blunted affect and social withdrawal, and a range of cognitive impairments (Kahn et al., 2015; Millan et al., 2012). As with BPD, both common and rare genetic variants create an additive risk of disorder development (Owen et al., 2011). Current research implies that the number of common variants involved in schizophrenia etiology might be in the thousands, but altogether only explain 50% of total SCZ variance (Giusti-Rodríguez & Sullivan, 2013), suggesting that these genetic vulnerabilities might interact with a stressful environment to cause SCZ-associated symptoms (DiLalla et al., 2017).

Autism spectrum disorders are characterized by deficits in social communication and interaction, as well as stereotypical or repetitive behavior (Lai et al., 2014). Their etiology is likewise considered to result from a highly complex interplay of genetic, environmental, and GxE influences. However, a recent study concluded that the majority of variance in ASD prevalence can be explained by genetic contributions (Bai et al., 2019), as opposed to environmental factors. It is likely that in this disorder, too, many common variants with moderate effects act together to form ASD risk instead of few large-effect variations (Chaste & Leboyer, 2012). Even though autism is considered a neurodevelopmental disorder, there are no viable biological tests that can aid diagnosis (DiLalla et al., 2017), and the exact relationship between genotype and phenotype remains an unresolved question due to the heterogeneity of symptoms (Chaste & Leboyer, 2012). A major focus of research has consequently been on finding the genetic underpinnings, with less emphasis on potential environmental causes (Chaste & Leboyer, 2012).

Regarding treatment of all of the psychiatric disorders highlighted above, it needs to be said that most current pharmacological approaches are aimed at the amelioration of symptoms, instead of tackling

the biological root causes that produce or maintain the disorder (Bhat et al., 2012). Yet even with proper medication, recurrence of symptoms is common, side effects occur frequently, and positive improvements sometimes only set in with long delays following the start of treatment. A significant improvement of therapeutics, however, can only be expected once we have a better understanding of the underlying pathophysiology of MDD, BPD, SCZ and ASD. Indeed, some authors even go as far as predicting that the identification of susceptibility genes and their interactions with environmental factors might eventually lead to targeted, individualized medicine (Insel & Scolnick, 2006).

In summary, when we consider the high likelihood of developing and immense suffering entailed in mental disorders across the world, combined with the altogether moderate effectiveness of current pharmacological treatments, the need to better understand the neurobiological factors contributing to their etiology becomes abundantly clear – to aid in the improvement of treatment methods and ultimately to be able to prevent disorder development. In this endeavor, one finding that has been consistently reproduced is the association of all four of the so far mentioned disorders with a variation in the *CACNA1C* gene, which this thesis will focus on.

CACNA1C

Definite gene identification in the etiology of psychiatric disorders has been hindered by their polygenetic and multifactorial nature, as well as an overlapping symptomology. However, the emergence of *CACNA1C* as a prominent risk gene for a range of diseases has proven to be one of the most statistically robust findings in the last decade of mental health research.

As outlined above, MDD, BPD, SCZ and ASD all stand out due to their relatively high heritability. Nonetheless, these estimates are only starting points in the search for the root causes of disease, as the fact of a high share of genetic components in disease etiology does not specify which genes exactly are responsible for the occurrence of a neuropsychiatric disorder.

In 2001, the completion of the “Human Genome Project” paved the way for genome-wide association studies (GWAS) on genetic variations and enabled the discovery of candidate genes for complex diseases (Uitterlinden, 2016). After decades of slow starts in psychiatric disease research, GWAS can now investigate thousands of genetic variations in a large number of probands, and in 2017 more than 50.000 samples had already been studied (Visscher et al., 2017). This type of study tests allele frequencies between patient and control groups and is uniquely equipped to uncover causative associations between common genetic variants like single-nucleotide polymorphisms (SNP) and traits in samples from large populations (Mullins & Lewis, 2017), providing empirical data for the validation of theoretical models, which has fundamentally changed the understanding of genetic disease

architecture (Uitterlinden, 2016). The effect size of individual polymorphisms uncovered in this process is usually relatively small, but collectively, they can account for e.g. one third of the variance in SCZ prevalence (Heyes et al., 2015). By leveraging the results obtained in GWAS, the exact mechanisms and pathways by which SNPs act to confer disorder risk can subsequently be examined in more detailed and candidate gene-specific experimental approaches.

An SNP is the substitution of one single nucleotide that occurs with a probability of more than 1% in the genome of a given population (Heyes et al., 2015), e.g. an adenine nucleotide (A-allele) in the place of a guanin nucleotide (G-allele) in one part of the DNA sequence. Most of the risk SNPs identified in GWAS are located in intronic, regulatory regions of the DNA and are thus not part of the coding sequence of a specific gene. In these cases, the "closest" possible gene is reported (Geschwind & Flint, 2015), as in the instance of SNP rs1006737 (minor risk allele A vs. G) and the *CACNA1C* gene, one of the first and most replicated genetic findings in psychiatry (Moon et al., 2018). Its first association with psychiatric disorders, namely BPD, was reported in a combined analysis of two GWAS by Sklar et al. in 2008, but was soon supported by independent studies of different BPD population samples (Ferreira et al., 2008; Sklar et al., 2011; Witt et al., 2014). In the years following this discovery, rs1006737 and other intronic *CACNA1C*-associated SNPs were linked to MDD (Casamassima et al., 2010; Rao et al., 2016), SCZ (Bigos et al., 2010; Nyegaard et al., 2010; Zhu et al., 2019) and ASD (Li et al., 2015a; Li et al., 2015b) across a variety of human populations. Apart from these GWAS, other authors have further implicated *CACNA1C* and cemented the relevance of calcium channels in cross-disorder analyses (Casamassima et al., 2010; Dao et al., 2010; Green et al., 2010; Heyes et al., 2015; Liu et al., 2011).

Biological function

The *CACNA1C* gene is located on chromosome 12 and encodes the pore-forming α_{1c} subunit of the L-type voltage-gated calcium channel (LTCC) $Ca_v1.2$ (Bhat et al., 2012).

Voltage-gated calcium channels primarily regulate the depolarization-induced calcium entry into the cell and can generally be categorized by whether they are activated by high voltage or low voltage changes in membrane potential (Simms & Zamponi, 2014). L-type calcium channels (Ca_v1 family) encompass four different channel proteins, $Ca_v1.2$-$Ca_v1.4$, and belong to the high-voltage-activated category, along with P/Q-type, N-type and R-type channels (Berger & Bartsch, 2014; Heyes et al., 2015). LTCCs are distinguished by their slow voltage-dependent inactivation and high sensitivity to organic calcium channel agents like dihydropyridine-agonists and antagonists (Catterall, 2011; Simms & Zamponi, 2014). However, only the $Ca_v1.2$ and $Ca_v1.3$ proteins (encoded by *CACNA1C* and *CACNA1D*, respectively) are assumed to be relevant to the neuronal membranes in the central nervous system (CNS) and the excitable cells in the heart (Casamassima et al., 2010).

Category	Gene	Protein	Physiological name
HVA	*CACNA1S*	$Ca_v1.1$	L-type
	CACNA1C	$Ca_v1.2$	
	CACNA1D	$Ca_v1.3$	
	CACNA1F	$Ca_v1.4$	
	CACNA1A	$Ca_v2.1$	P/Q-type
	CACNA1B	$Ca_v2.2$	N-type
	CACNA1E	$Ca_v2.3$	R-type
LVA	*CACNA1G*	$Ca_v3.1$	T-type
	CACNA1H	$Ca_v3.2$	
	CACNA1I	$Ca_v3.3$	

Table 1: Overview of voltage-gated calcium channel subunits, including gene name, protein names and names used in physiological discovery of the channels (adapted from Heyes et al., 2015). HVA = high-voltage-activated calcium channels. LVA = low-voltage-activated calcium channels.

A functional $Ca_v1.2$ channel is comprised of the following four subunits: transmembrane α_{1c}, auxiliary $\alpha_2\delta$, intracellular β and calmodulin. The α_{1c} subunit is encoded by the *CACNA1C* gene and regulates the biophysical and pharmacological $Ca_v1.2$ channel characteristics, such as voltage dependency and binding sites for drugs and second messengers. Whereas $\alpha_2\delta$, β and calmodulin fine-tune the channel function (Bhat et al., 2012; Catterall, 2011), α_{1c} forms the conduction pore through which calcium enters the cell. Calcium constitutes an essential neuronal signaling molecule, and a plethora of physiological processes is triggered by the elevation of intracellular calcium concentrations – muscle and heart contraction, hormone secretion, cell growth and apoptosis, and gene transcription (Berridge et al., 2000; Carafoli, 2002), to name but a few. These processes are contingent on the proper conversion of electrical activity into biochemical events in excitable cells, as mediated by LTCCs (Gomez-Ospina et al., 2006).

In the CNS, LTCCs are mainly found in postsynaptic dendritic spines, cell somata as well as in presynaptic axonal terminals, although the percentage of $Ca_v1.2$ channels in this latter location is relatively small (Leitch et al., 2009). In fact, at regular neuron terminals, there is a higher presence of voltage-gated calcium channels belonging to the Ca_v2 and Ca_v3 family (see Table 1). However, Ca_v1 channels cluster at ribbon synapses, where they are involved in neurotransmitter release by triggering the fusion of the synaptic vesicles and the cell membrane (Catterall, 2011), following the membrane depolarization and subsequent calcium influx caused by an incoming action potential. Postsynaptically, on the other hand, the close proximity of $Ca_v1.2$ channels to the cell soma puts them in an ideal place to control synapse to nucleus signaling, as compared to other voltage- or ligand-gated channels

(Catterall, 2011), and thereby activating pathways that regulate gene transcription (Gomez-Ospina et al., 2006). Calcium influx into the post-synapse via LTCCs also triggers the secretion of important growth factors, which have an essential role in synaptic plasticity (Cunha et al., 2010; Kolarow et al., 2007). Since its initial discovery, several studies have ascertained the relevance of postsynaptically located *CACNA1C* for memory formation and learning processes (Bhat et al., 2012; Leitch et al., 2009), and $Ca_v1.2$ channels have also previously been associated with early brain development (Heyes et al., 2015). Consequently, by translating electrical signals on membrane level to diverse downstream molecular cascades and intracellular responses, $Ca_v1.2$ channels influence the correct functioning of many brain regions related to psychiatric disease pathophysiology, as has been shown via the administration of LTCC agonists and antagonists, as well as in genetically altered animal models (Bhat et al., 2012; Catterall, 2011).

CACNA1C expression levels in general are highly species- and tissue-dependent and often affected by pathophysiological conditions (Bhat et al., 2012; Kabir et al., 2017c). While within the CNS, $Ca_v1.2$ channels show highest expression in the hippocampus (HC) and cerebellum (Splawski et al., 2004), and $Ca_v1.2$ is generally known to be the primary LTCC in the mammalian brain (Obermair et al., 2008), it is also expressed in many additional tissues: in cardiac and smooth muscle, as well as endocrine cells, such as the pancreas and adrenal glands (Casamassima et al., 2010; Striessnig et al., 2014). In the endocrine system, for example, LTCCs mediate the secretion of hormones (Catterall, 2011). By and large, calcium has proven to be one of the most relevant signaling molecules for various organisms including, but not limited to, humans (Splawski et al., 2004). Its regulation via $Ca_v1.2$ channels is essential for many processes, and an imbalance in expression can have highly detrimental effects. Regarding the brain, for instance, an excessive influx of calcium into the cell has been hypothesized to underlie processes associated with cognitive decline, like brain ageing and dementia (Navakkode et al., 2018). In the cardiac muscle, too, a balanced distribution of the predominant $Ca_v1.2$ channels is key, as both increases and decreases have been linked to heart disease, and research on mice has revealed heart failure and consequently high lethality in heterozygous (HET), but especially homozygous *Cacna1c* knockouts (Striessnig et al., 2014).

With most reported *CACNA1C* risk alleles being located in intronic, non-coding regions of the gene, it is assumed that SNPs like rs1006737 might regulate the gene's expression, rather than its function (Eckart et al., 2016; Harrington & Allen, 2014), but it is still not fully understood whether this influence is constant across different tissues and during an individual's lifespan (Bhat et al., 2012). What can be said, though, is that an inappropriate expression of $Ca_v1.2$ channels has been shown to produce negative effects on various neurobiological processes and symptoms of psychiatric disorders. Preclinical rodent studies, which will be highlighted in more detail further below, have already allowed

us a glimpse into which molecular and cellular pathways might be affected by $Ca_v1.2$ expression anomalies (Kabir et al., 2017c), but the transcriptional control of *CACNA1C* appears to be highly complex (Simms & Zamponi, 2014), and even small changes in expression and activity might result in e.g. cardiac arrhythmias or phenotypes associated with psychiatric disease (Striessnig et al., 2014).

Enabled by GWAS, we have been able to make remarkable discoveries via targeted approaches in individual research projects, but at the same time they underscore the complexity of mental conditions (Geschwind & Flint, 2015). Exactly how risk SNPs like rs1006737 influence allelic transcription levels still needs to be clarified, and how these changes, in turn, are associated with psychiatric disease still remains, in large parts, a mystery (Striessnig et al., 2014). What becomes abundantly clear, however, is that the complex biological mechanisms by which *CACNA1C* exerts its influence on so many diverse conditions cannot be explained in a disorder-specific manner. The key, therefore, might lie outside of the boundaries of our current diagnostic classification systems and in a different perspective on disorder-related phenotypes.

Behavioral domains

To pave the way for improved therapy and potential prevention of neuropsychiatric diseases, the molecular underpinnings need to be elucidated first. Here, independent studies and meta analyses of GWAS have suggested large genetic overlaps between different diagnoses (Geschwind & Flint, 2015; Witt et al., 2014), such as in MDD and BPD (Craddock & Sklar, 2013; Rowland & Marwaha, 2018), as well as between affective disorders and SCZ (Berrettini, 2005; Eckart et al., 2016; Moskvina et al., 2008). In addition, there is ample evidence from epidemiological and neuroimaging studies that SCZ susceptibility genes also confer risk to ASD, pointing towards shared risk loci and neurobiological pathways (Li et al., 2015b).

Unfortunately, the diagnostic instruments currently employed in clinical practice rely heavily on symptom clusters, observable behavioral traits and patients' own descriptions of their experiences (Gottesman & Gould, 2003). This system promotes communication between clinical practitioners (O'Donovan & Owen, 2016) but is insufficiently equipped to consider aspects such as comorbidity, and the high heterogeneity in symptoms and recovery rates (Hasler et al., 2004; O'Donovan & Owen, 2016). The progress in understanding mental disorders and identification of responsible genes has consequently been slow due to the missing link from diagnostic classification to pathophysiological mechanisms in disease (Calabrò et al., 2020), which has led to patients not getting adequate treatment or not showing significant improvements through medication (Kalueff et al., 2015). With improvements to diagnostic categories in the new version of the DSM being judged as insufficient, and

hopes not high for a research-oriented replacement in the near future (Möller et al., 2015), the shortcomings of current diagnostic instruments call for a new classification system that reflects the complexity of the brain and focuses on shared etiology (Owen et al., 2011). As biological risk factors do not adhere to manmade diagnostic boundaries, nosology should rather be informed by the causes of disease and common intermediate phenotypes to improve our understanding and ultimately help the affected patient (Geschwind & Flint, 2015; Green et al., 2010; Rao et al., 2016).

Trend endophenotypes

The endophenotype approach, originally presented in the context of psychiatric disorders by Gottesman and Shields in 1972, attempts to deconstruct psychiatric diagnoses and create more homogenous subgroups by defining intermediate phenotypes that are closer to the effect of susceptibility genes than a specific diagnosis (Berrettini, 2005; Geschwind & Flint, 2015). This line of action is consistent with the fact that several neuropsychiatric conditions have been shown to share intermediate behavioral phenotypes, such as impairments in social interactions and communication deficits (Bai et al., 2019; Cattane et al., 2018; DiLalla et al., 2017; Hirschfeld et al., 2000; O'Donovan & Owen, 2016; Sanchez-Moreno et al., 2009) or cognitive dysfunction, a core feature of many mental disorders (Bearden & Freimer, 2006; Cattane et al., 2018; DiLalla et al., 2017; Hasler et al., 2004; Millan et al., 2012; Moon et al., 2018). Next to these behavioral measures, potential neurobiological examples for intermediate phenotypes include functional connectivity deficits and neurodevelopmental abnormalities (Bai et al., 2019; King & Lord, 2011; Owen et al., 2011) structural brain alterations such as in hippocampal volume (Cirillo & Seidman, 2003; Hasler et al., 2004), but also electroencephalographic anomalies or aberrant adult hippocampal neurogenesis (Iacono, 2018; Kabir et al., 2017c).

The risk gene *CACNA1C* has repeatedly been implicated in behavioral domains relevant for neuropsychiatric conditions, such as emotion and mood, motivation, substance abuse and social and cognitive functioning (Kabir et al., 2016; Moon et al., 2018). The following paragraphs will highlight several key components of cognition and social behavior, describe how these are affected in mental disorders and to what degree they have been associated with *CACNA1C* in studies on human subjects. With an emphasis on cognitive abilities, this doctoral thesis will then explore the impact of *Cacna1c* on the learning and social behavior of rats as a model species, in the context of sex, different environmental rearing conditions and related findings in both rats and mice.

Cognition

In 2010, the National Institute of Mental Health proposed the utilization of behavioral domains to explore the impact of genetic polymorphisms on neuronal function and circuits in the Research Domain

Criteria Framework with the aim of finding new ways to classify mental illnesses to overcome categorical limitations of current diagnostic system (Insel et al., 2010). This approach allows for the study of cognition-related phenotypes that are also present in undiagnosed relatives of affected patients to elucidate at-risk states and symptoms. Examples of such phenotypes include, but are not restricted to, core components of cognition like verbal learning and memory, executive function and working memory (Millan et al., 2012) which have been shown to be impaired in most major mental disorders.

Whether we need to remember where we put our keys before leaving the house, what the name of an acquaintance is, to follow a conversation or to plan a vacation, almost every intentional human behavior requires the encoding, retrieval and prioritization of information (Miller, 2000). When applied correctly, these actions increase the probability of success in social, academic and work areas of life. Studies on affective and psychotic disorders often focus on the positive or affective symptoms. However, while the degree of impairment may vary depending on diagnosis (Krug et al., 2010), all major neuropsychiatric disorders are characterized by at least one type of cognitive alteration, often associated with diminished functional outcomes and quality of life. For example, in bipolar research, cognitive impairments went from being largely discounted to one of the major defining aspects of the disorder (Cipriani et al., 2017), affecting all cognitive functions except verbal and general intelligence (Rolstad et al., 2016). Similarly, attention, memory and executive function deficits are now seen as core features of MDD (Hammar & Ardal, 2009) and SCZ (Bora, 2016; Manoach, 2016) where only around 30% of patients do not report cognitive symptoms (Atique-Ur-Rehman & Neill, 2019; Rund, 1998). Of note, several reports have found that during remission, cognitive impairments do not seem to be alleviated to the same degree as other symptoms and cripple patients even in euthymic states (Goldberg & Chengappa, 2009; Hammar & Ardal, 2009; Vöhringer et al., 2013). Next to diagnosed patients, cognitive dysfunction can also be observed in healthy first-degree relatives, indicating that these individuals might be at-risk of developing additional symptoms and could potentially benefit from early interventions (Kim et al., 2015; MacQueen & Memedovich, 2017).

Deficits in the cognitive domain can have highly detrimental consequences on an individual's ability to focus, their educational achievements, work performance, and social and professional integration (Atique-Ur-Rehman & Neill, 2019; Millan et al., 2012; Richardson & Adams, 2018), leading to limited options and a drastically reduced quality of life. Furthermore, impairments in cognitive abilities have been linked to MDD and BPD relapse and recovery rates (Atique-Ur-Rehman & Neill, 2019; Millan et al., 2012), as well as the presence of psychotic symptoms (MacQueen & Memedovich, 2017). In fact, in SCZ, cognitive deficits are the strongest predictor of functional outcomes (Turner & Burne, 2013). Unfortunately, whereas some symptoms like listlessness in MDD and hallucinations in SCZ can

definitely be improved by medication, current drug treatments have been found wanting for adequate mitigation of cognitive dysfunction (Hill et al., 2010; MacQueen & Memedovich, 2017; Millan et al., 2012). Together with the high prevalence and debilitating consequences of cognitive impairments, missing treatment options further highlight the importance of a better understanding of how the *CACNA1C* risk gene influences learning and memory on a behavioral level.

Declarative memory

The ability to select, encode, retain and retrieve information is essential for all components of cognition. In contrast to associative and procedural learning in non-declarative memory, however, it is believed that the recollection of facts (semantic memory) and events (episodic memory) in declarative memory cannot take place without conscious awareness (Millan et al., 2012). Humans are also generally able to remember their past, the approximate order of events and the contexts in which they were experienced. If this ability is compromised, the effect on daily functioning and further skill acquisition can be severe (Cirillo & Seidman, 2003). A popular test of declarative memory are verbal fluency paradigms, which require subjects to name as many unique items as possible within one semantic category.

In terms of *CACNA1C*, several studies have already linked risk variants of the gene to aberrations in declarative memory. A decade ago, the first study on the effect of *CACNA1C* on human cognition associated the risk allele with decreased verbal fluency in healthy males (Krug et al., 2010). Subsequent studies provided mixed results, with one study replicating the finding in depressed patients (Backes et al., 2014) while others found no effect of *CACNA1C* on episodic memory performance in healthy individuals (Erk et al., 2010; Erk et al., 2014a; Roussos et al., 2011) or those affected by BPD (Rolstad et al., 2016) and their first degree relatives (Arts et al., 2013; Erk et al., 2014b). Then again, impairments in SCZ patients carrying the minor *CACNA1C* allele were detected in a logical memory task (Hori et al., 2012). The discrepancy between these studies was hypothesized to be due to varying difficulty levels between tasks (Krug et al., 2014), a first indication that *CACNA1C* might have differential effects on learning and memory depending on experimental protocol. Poor general learning performance in healthy risk allele carriers was also observed by Dietsche and colleagues, although in their study, memory recall and recognition were unaffected by *CACNA1C* (Dietsche et al., 2014).

Cognitive control

Cognitive control refers the ability to plan and coordinate actions by distinguishing goal-relevant from irrelevant stimuli, inhibiting impulsive or inappropriate reactions and adjusting one's behavior to new circumstances. Declarative memory and cognitive control are connected, in that all intentional learning requires cognitive control (Miller, 2000). In research, "executive function" is often used as a

comprehensive term for goal-directed behavior, impulse control and cognitive flexibility (Millan et al., 2012), and is measured via tasks that require the allocation of attention, prioritization and the switching of cognitive strategies when environmental contingencies change (Soeiro-de-Souza et al., 2013).

Study	***CACNA1C* SNP**	**Phenotype**
Arts et al., 2013	rs1006737	NE verbal memory [BPD, 1st, H] NE working memory [BPD, 1st, H] ↓ general cognitive performance [BPD] NE general cognitive performance [1st, H]
Backes et al., 2014	rs1006737	↓ verbal fluency [MDD]
Cosgrove et al., 2017	rs2007044	↓ working memory [SCZ]
Dietsche et al., 2014	rs1006737	↓ episodic memory (acquisition) [H] NE episodic memory (recall, recognition) [H]
Erk et al., 2010	rs1006737	NE episodic memory [H]
Erk et al., 2014a	rs1006737	NE episodic memory [H]
Erk et al., 2014b	rs1006737	NE episodic memory [1st]
Hori et al.,2012	rs1006737	↓ logical memory [SCZ] NE logical memory [H] NE executive function [SCZ, H] NE working memory [SCZ, H] NE general cognitive performance [H]
Krug et al., 2010	rs1006737	↓ verbal fluency [H]
Krug et al., 2014	rs1006737	NE episodic memory [H]
Paulus et al., 2014	rs1006737	NE working memory [H]
Rolstad et al., 2016	rs1006737	NE episodic memory [BPD, H] NE executive function [BPD, H] NE working memory [BPD, H] NE general intelligence [BPD]
Roussos et al., 2011	rs1006737	NE spatial memory [H] NE executive function [H]
Soeiro-de-Souza et al., 2013	rs1006737	↓ executive function [BPD] NE executive function [H] ↓ working memory [BPD] NE working memory [H]
Sykes et al., 2019	rs1006737 and rs2007044	↓ reversal learning accuracy [H]
Takeuchi et al., 2018	rs10254582	NE working memory [H]
Zhang et al., 2012	rs1006737	↓ working memory [SCZ] ↓ working memory [H] ↑ working memory [BPD]

Table 2: *CACNA1C* studies on selected components of human cognition. ↓ = decreased. ↑ = increased. NE = no effect. 1st = first-degree relatives of diagnosed patients. BPD = bipolar disorder. H = healthy subjects. MDD = major depressive disorder. SCZ = schizophrenia.

CACNA1C associations with executive function are dependent on diagnosis. Hori and colleagues did not observe genotype-dependent deficits in the Wisconsin Card Sorting Test in either SCZ patients or healthy controls (Hori et al., 2012), yet one of the first studies to examine *CACNA1C* in bipolar patients

found SNP rs1006737 to be associated with poor cognitive flexibility in both the Wisconsin Card Sorting Test and the trail making test in BPD, but not in undiagnosed individuals (Soeiro-de-Souza et al., 2013). Two further studies also failed to discover a link of the *CACNA1C* risk allele with executive function in healthy populations (Rolstad et al., 2016; Roussos et al., 2011). Then again, the minor variant of rs1006737 was associated with poor reversal learning (RL) performance in healthy subjects during a probabilistic RL task (Sykes et al., 2019) and in a very recent study, a different *CACNA1C* risk allele was hypothesized to play a role in the impulsive behavior often seen in SCZ (Calabrò et al., 2020).

Working memory

The term working memory (WM) refers to a limited-capacity network that can hold and manipulate information e.g. to evaluate a selection for goal-relevant items (Millan et al., 2012). As such, it is closely connected to executive function and an essential companion to declarative memory, as all information passes through working memory first for appraisal before being committed to long-term storage (Wallace et al., 2015). WM can be measured via tasks that require the simultaneous maintenance of several distinct items, like a sequence of numbers. Popular examples include the n-back test and the digit span paradigm (Berrettini, 2005).

Various studies have reported no association between *CACNA1C* risk alleles and working memory performance in healthy participants (Arts et al., 2013; Hori et al., 2012; Paulus et al., 2014). BPD patients were found to perform worse in the digit-span paradigm than healthy controls, consistent with previous reports on disorder impairments, but this effect was observed irrespective of *CACNA1C* genotype (Rolstad et al., 2016). By contrast, another study reported poorer WM in healthy risk allele carriers and contrary results depending on the specific diagnostic group, with reduced capabilities in SCZ patients and improved performance during the 1-back task in BPD probands (Zhang et al., 2012). Then again, no effect of risk allele on WM performance in SCZ was found by Hori and colleagues (Hori et al., 2012), but worse digit-span results were obtained in BPD patients homozygous for the risk allele (Soeiro-de-Souza et al., 2013), yet not at all in their first-degree relatives (Arts et al., 2013). Next to these conflicting results on rs1006737, two other intronic *CACNA1C* risk alleles have yielded opposing results, with one showing no correlation with impaired WM (Takeuchi et al., 2018) and the other associated with poor WM performance in several samples of SCZ patients during a spatial WM test and various measures from the MATRICS battery (Cosgrove et al., 2017). The exact mechanism accounting for these discrepancies is currently unknown but has been hypothesized to depend on differences in paradigm protocols and insufficient sample sizes (Takeuchi et al., 2018).

Apart from these construct-specific results, *CACNA1C* has generally been linked to impaired cognitive performance and overall memory function in some studies (Arts et al., 2013; Dietsche et al., 2014), but

not in others (Hori et al., 2012; Rolstad et al., 2016; Roussos et al., 2011), indicating that the effects may be small in size (Rolstad et al., 2016), or that *CACNA1C* potentially interacts with additional factors on intermediate neurobiological substrates (Ou et al., 2015). As impairments of declarative memory, executive functioning and working memory affect most patients burdened by psychotic and mood disorders, as well as by ASD (Krug et al., 2010), and are heavily linked to functional outcomes, disorder course and treatment response (Hammar & Ardal, 2009), it is imperative to decipher the underlying factors contributing to these deficits.

Social behavior

Like cognition, social behavior in humans is a complex concept that interacts with and influences many different parts of our lives, from social interactions and the bonds we form with friends and family to hierarchical relationships at work, appropriate communication with peers, and complex concepts like empathy and prosocial behavior. Additionally, the quality of our social relationships is vital for our mental health and can ameliorate the negative consequences of stress (Kendler et al., 2005). Appropriate social interaction and communication is highly context-specific and requires the correct recognition and interpretation of social signals like words, tone of voice, gestures and facial expressions, as well as the selection and execution of an adequate response (Holmes, 2019). When these intricate abilities are impaired, they prohibit individuals from activities involving social learning and sharing, collaborating and generally participating in society (Porcelli et al., 2019).

Deficits in social behavior are common in a range of neuropsychiatric disorders (Meyer-Lindenberg & Tost, 2012) and include social withdrawal, perception errors, social anxiety, aberrant social cue identification and information processing, and social memory impairments. In MDD, the dysfunction of social behaviors is a persistent symptom and has been recognized as a core identifier of depression (Hirschfeld et al., 2000). Patients frequently experience difficulty in keeping up reciprocal cooperation, and display less motivation to interact with others (Porcelli et al., 2019). In BPD, social dysfunction appears to be similar, although expressed in a potentially more pronounced fashion than in MDD (Sanchez-Moreno et al., 2009). Patients with a diagnosis of SCZ and their families often suffer from the social disability in the affected individual that often becomes manifest in childhood and adolescence, long before a diagnosis is made (Porcelli et al., 2019). In fact, both SCZ and ASD are characterized by a marked deviation in social behavior and communication and share difficulties in social information processing (Eack et al., 2013; Pu et al., 2019). Notably, the *CACNA1C*-associated Timothy syndrome (TS) is likewise defined by social communication impairments (Splawski et al., 2004), and there are even disorders prominently featuring hypersociability, which coupled with prevalent social withdrawal highlights the broad spectrum of potential alterations in the social domain (Porcelli et al., 2019).

The inability to accurately process social signals reduces the capacity to integrate cognitive and affective skills in order to adapt to social contexts. Furthermore, it can cause inappropriate behavior, making exclusion from social groups more probable (Porcelli et al., 2019; Wöhr et al., 2019). Affected individuals miss out on naturally rewarding positive social interactions and experience social pain from isolation which can result in a vicious circle, as social withdrawal is known to exacerbate social dysfunction and can eventually lead to consequences like decreased work performance, unemployment, disability, and the aggravation of depressive symptoms and suicidality (Porcelli et al., 2019; Schafer & Schiller, 2018). Regrettably, and as in cognitive dysfunction, the effective treatment of social deficits still constitutes an unmet need (Ferretti & Papaleo, 2019; Young et al., 2009).

Affiliation & attachment

Affiliation and sociability refer to an individual's engagement in friendly interactions and social bonding, both of which are contingent on social motivation. In several disorders, the phenomenon of social anhedonia is highly prevalent, defined by a significantly diminished interest in social affiliation. This symptom is common in BPD patients, who often appear to have less contact with friends and family than undiagnosed subjects (Kupferberg et al., 2016; Sanchez-Moreno et al., 2009). In SCZ, social withdrawal is often one of the first obvious symptoms to appear in at-risk populations (Young et al., 2009), and might even trigger full SCZ in vulnerable individuals, although the reduction in sociability can be only mildly expressed, too (Porcelli et al., 2019). By contrast, aberrant social interaction is one of the key features of the ASD triad (Lai et al., 2014). Autistic children often fail to respond appropriately – or at all – to the initiation of social contact, and diagnosed adults often experience difficulties in developing and maintaining social relationships (DiLalla et al., 2017). In MDD, which is heavily associated with social anhedonia, affected patients derive less enjoyment from interactions with friends and family (Dere et al., 2010) and often give others the idea that they are not interested in social contact. At the same time, however, they are more sensitive to rejection and often react in a hostile manner, followed by social withdrawal, if instances of exclusion occur (Kupferberg et al., 2016).

In *CACNA1C* risk allele carriers, no direct effects on social interaction have been found to date. However, in healthy male participants, the risk variant was implicated in lower extraversion scores and greater anxiety (Roussos et al., 2011). These traits may conceivably impair an individual's desire to seek out social contact and consequently result in reduced interaction. A significantly increased interpersonal sensitivity – a score indicative of discomfort during social interactions and negative expectations about personal relations – was furthermore observed in healthy risk allele carriers by Erk and colleagues (Erk et al., 2010). Compatible with this, greater hostility and reduced emotional intelligence were seen in a more recent study on female risk allele carriers (Takeuchi et al., 2018), further strengthening the hypothesis of *CACNA1C* involvement in impaired social interaction.

Social communication

Social communication requires the recognition and correct interpretation of social stimuli. In humans, these cues can most often be found in the facial expression of another person, but the demonstration of emotional states and intentions also encompasses gestures, speech, prosody and playful behavior. In neuropsychiatric disorders, and especially in ASD, many of these communicative functions are impaired, as demonstrated by poor eye contact, atypical gestures and verbal alterations (DiLalla et al., 2017; Iacono, 2018), and by difficulties in interpreting body language and facial expressions (Cattane et al., 2018). The latter is also observed in patients of BPD and SCZ (Nieratschker et al., 2015). As face perception is one of the primary means of identifying fellow human beings, this results in a great handicap for the affected individuals (Young et al., 2009). Autistic children tend to ignore an initiation of playful behavior (DiLalla et al., 2017), another indication that the understanding of social cues is reduced. Speech prosody, as another example, can give hints towards the speaker's emotional state, and is often impaired in patients suffering from a major neuropsychiatric disorder (Koch et al., 2019). In a similar vein, general verbal and language deficits like disorganized speech or increased speech latency during dialogue are furthermore displayed by MDD (Kupferberg et al., 2016) and SCZ patients (Manoach, 2016). In fact, a delayed language acquisition in children can be one of the initial signs that predispose for mental illness later in life (Harrington & Allen, 2014; Koomar & Michaelson, 2020).

Study	***CACNA1C* SNP**	**Phenotype**
Dima et al., 2013	rs1006737	NE facial emotion recognition [BPD, H]
Erk et al., 2010	rs1006737	↑ interpersonal sensitivity [H]
Nieratschker et al., 2015	rs1006737	↓ facial emotion recognition (latency) [H] NE facial emotion recognition (accuracy) [H]
Roussos et al., 2011	rs1006737	↓ extraversion [H] ↑ trait anxiety [H]
Soeiro-de-Souza et al., 2012	rs1006737	↓ facial emotion recognition [BPD] NE facial emotion recognition [H]
Takeuchi et al., 2018	rs1024582	↓ emotional intelligence [H] ↑ hostility [H]

Table 3: *CACNA1C* studies on selected components of human social behavior. ↓ = decreased. ↑ = increased. NE = no effect. BPD = bipolar disorder. H = healthy subjects.

Many findings implicate *CACNA1C* in aberrant social cue reception, like facial emotion recognition deficits. BPD patients carrying the risk allele, for example, display impaired facial affect recognition compared to non-risk BPD patients and healthy controls (Soeiro-de-Souza et al., 2012). Whereas no alterations in this domain were detected in another study on BPD (Dima et al., 2013), slower response times at normal accuracy were displayed by healthy participants during the identification of facial emotions in the "Reading the Mind in the Eyes" test (Nieratschker et al., 2015). Next to these behavioral recognition measures, Krautheim and colleagues found social outgroup emotion processing

to be influenced by *CACNA1C* in a brain activation study (Krautheim et al., 2018), and on top of this, the gene has also been associated with altered language development (Splawski et al., 2004) and production (Casamassima et al., 2010) in humans.

All in all, it becomes clear that social and cognitive impairments in mental disorders can take many forms, and that the distinction between the two highly correlated domains is not easy (Young et al., 2009), seeing that adequate social behavior also depends on intact executive functions like working memory and impulse control (Porcelli et al., 2019). With the manifold negative consequences patients of neuropsychiatric disorders need to face in daily life, the necessity of a better etiological understanding of these deficits cannot be dismissed. Clearly, the effect of *CACNA1C* polymorphisms on human cognitive abilities and social behavior is not straightforward. However, translational research may hold the key to unravel the underlying mechanisms and eventually enable a better identification and treatment of *CACNA1C*-related behavioral phenotypes in these domains.

Cacna1c research in mice

One of the many benefits of the behavioral domain concept is that it can be readily applied to and consequently increase the translational value of preclinical animal models (Kalueff et al., 2015). These, in turn, can help to identify pathways by which genes act on a given phenotype and aid in the development of quantitative, biologically valid tests to guide diagnosis (Glahn et al., 2014).

As has become evident in the previous sections, the results regarding *CACNA1C* effects on human social and cognitive behavior are quite inconsistent. Disorder research in human subjects comes with many challenges. From working with retrospective data on experiences that can fall prey to forgetting or reporting bias caused by current mood (Hardt & Rutter, 2004) and the ethical limitations for the control of potentially confounding factors in an individual's life, to the multitude of genetic variations in one genome, many impediments prevent human trials from drawing definite causal conclusions. A common approach to circumvent these challenges is to investigate specific intermediate phenotypes associated with neuropsychiatric disorders in animal models that allow for the monitoring and systematic variation of experimental conditions like genetic manipulation or environmental stimuli. Rodents have proven especially useful as they reproduce quickly, are capable of many abilities relevant for human behavior and their DNA sequence provides orthologues for 80-90% of human genes (Mogil, 2019). Mice, in particular, are an established species that dominates preclinical research studies and even though technically speaking, there are no "depressive" or "schizophrenic" mice, they provide valid means to study intermediate social and cognitive phenotypes with relevance to neuropsychiatric disorders (Keeler & Robbins, 2011; Nestler & Hyman, 2010; Salgado & Sandner, 2013).

Like in the human CNS, $Ca_v1.2$ is highly expressed in the mouse brain (Sinnegger-Brauns et al., 2009), providing various labs around the world with the opportunity to generate mutant mouse models with relevance to *CACNA1C* in humans (see Table 4). As SNP rs1006737 lies in a non-coding region of the gene, like several other *CACNA1C* risk alleles (Bhat et al., 2012), it is assumed that these genetic variants affect *CACNA1C* expression, rather than influencing the actual function of the expressed $Ca_v1.2$ protein (Heyes et al., 2015). As both increases and decreases of *CACNA1C* mRNA have been reported in risk allele carriers, however, there is no clear-cut answer as to the direction of the influence on $Ca_v1.2$ function so far (Bigos et al., 2010; Gershon et al., 2014; Roussos et al., 2014; Yoshimizu et al., 2015). Studies on the effects of *Cacna1c* in mice have therefore employed varying methods of genetic manipulation, from region-specific knockouts over haploinsufficient models to knock-in mutants (Bader et al., 2011; Kabitzke et al., 2018). Since mice with a homozygous ablation of *Cacna1c* have been shown to be embryonically lethal (Seisenberger et al., 2000), most findings are based on constitutive HET deletions or region-specific conditional knockout lines e.g. in the prefrontal cortex (PFC) (Kabir et al., 2017b) or the anterior cingulate cortex (ACC) (Jeon et al., 2010). Both HET and conditional knockout models have been shown to exhibit behavioral alterations with relevance to human disorders (Kabir et al., 2016; Moon et al., 2018) which will be described in more detail below.

Cognitive abilities in *Cacna1c* mice

Humans are constantly employing their cognitive abilities to integrate information, select the relevant parts for their own goals and adapt their behavior to changing situational contexts. In rodents, similar abilities are necessary to avoid predators, find food and shelter, and to secure potential mating partners and eventual propagation. Indeed, episodic-like memory qualities have been postulated for many species, including mice (Mogil, 2019). The basis of many methods investigating mechanisms of human cognition in this species is spatial navigation, which is essential for the daily life of rodents and, like episodic memory in humans, is considered to be hippocampus-dependent (Morellini, 2013). One of the most popular paradigms in this context is the Morris water maze (MWM) that requires mice to find a submerged platform in a basin filled with opaque water (Morris, 1981). Several authors have ascertained that a region-specific knockdown of *Cacna1c* in the forebrain results in impaired learning in the MWM. Mice with conditional forebrain deletions of *Cacna1c*, for instance, showed drastically altered learning curves compared to controls (Moosmang et al., 2005b) and remote recall deficits in a probe trial administered 30 days after the learning period had ended (White et al., 2008). In addition, more or less striking impairments in other water-based mazes were obtained depending on task difficulty and timing of *Cacna1c* knockout (Dedic et al., 2018; Kabir et al., 2017a; Temme et al., 2016). By contrast, a knock-in model of *Cacna1c* showed intact spatial learning (SL) in the MWM (Bader et al., 2011) and in the procedural T-maze task (Kabitzke et al., 2018). Regarding recognition memory, which

is usually assessed in the novel object recognition test (NOR), findings are similarly mixed, with a region-specific deletion in the ACC not affecting performance in one study (Jeon et al., 2010) but increased levels of constitutive *Cacna1c* expression causing decreased recognition abilities in both male and female mice in another experiment (Zanos et al., 2015). On top of these genetic approaches, the involvement of calcium channels in memory has been further indicated by studies employing pharmacological LTCC blockers like nimodipine, which enhanced declarative learning (Casamassima et al., 2010) and exerted a protective influence on cognitive abilities in aged animals (Ingram et al., 1994).

To elucidate the divergence in human findings regarding executive function, a handful of studies have also investigated *Cacna1c* mice in paradigms that require cognitive flexibility and automatic response inhibition (Keeler & Robbins, 2011; Wöhr & Scattoni, 2013). These tests can measure compulsive behavior and inappropriate reactions to negative feedback by changing reward contingencies, or via extinction trials in fear conditioning (Kabir et al., 2016). Unfortunately, little attention has been allocated to reversal learning in translational *Cacna1c* models so far. Only one lab investigated the consequences of a forebrain-specific *Cacna1c* deletion on cognitive flexibility in mice and found antithetic effects depending on the stage of life at which the knockout procedure was performed. Notably, significant difficulties in water cross maze RL were detected in developmental knockouts, whereas in animals with *Cacna1c* reduction in adulthood, cognitive flexibility was even enhanced (Dedic et al., 2018). More compelling evidence comes from studies using the knock-in Timothy syndrome mouse TS2-neo. While Kabitzke and colleagues did not find significant RL deficits in male TS2-neo mice in a procedural T-maze (Kabitzke et al., 2018), striking perseverative tendencies were evident in the extinction of fear memories, and performance in the Y-maze task and MWM in another study, where TS2-neo mice spent considerably more time in the previously relevant target quadrant of the basin after the escape platform was moved (Bader et al., 2011).

In terms of WM, constitutive *Cacna1c* HET mice were found not to be impaired in the spontaneous alteration test, which is usually employed to measure working memory (Zanos et al., 2015), unless they were exposed to chronic unpredictable stress 1-2 days prior to assessment. In this, however, they did not differ from wildtype (WT) controls (Bavley et al., 2017).

Social behavior in *Cacna1c* mice

Next to cognitive abilities, mice also display an array of social behaviors, such as living together in groups in large burrows, communal nesting and olfactory, as well as acoustic communication with conspecifics (Ellenbroek & Youn, 2016). These natural tendencies are the reason the species is often studied in the context of translational research on human social interaction.

Mouse model	Study	Phenotype
Cacna1c HET	Bader et al., 2011	NE sustained social preference (home cage assay)
Cacna1c HET	Zanos et al., 2015	NE working memory ↑ object recognition (in aged animals)
Cacna1c HET	Bavley et al., 2017	NE working memory
Cacna1c HET	Dedic et al., 2018	NE social preference
Cacna1c HET (forebrain) during adulthood	Dedic et al., 2018	NE social preference
Cacna1c-knockout (forebrain)	Moosmang et al. 2005	↓ spatial learning
Cacna1c-knockout (forebrain)	White et al. 2008	NE spatial learning ↓ spatial learning (long-term)
Cacna1c-knockout (forebrain)	Kabir et al., 2017a	↓ spatial learning NE working memory ↓ social preference
Cacna1c-knockout (forebrain) during development	Dedic et al., 2018	↓ spatial learning ↓ reversal learning ↓ social preference
Cacna1c-knockout (forebrain) during adulthood	Dedic et al., 2018	NE spatial learning NE spatial learning (long-term) ↑ reversal learning NE social preference
Cacna1c-knockdown (adult PFC)	Kabir et al., 2017a	NE spatial learning NE working memory ↓ social preference
Cacna1c-knockout (CNS)	Temme et al., 2016	NE spatial learning (MWM) ↓ spatial learning (MWM with limited cues)
Cacna1c-knockout (CNS)	Dedic et al., 2018	↓ social preference
Cacna1c-knockout (ACC)	Jeon et al., 2010	NE object recognition ↓ social fear learning
Cacna1c-knockout (NAcc)	Terrillion et al., 2017	NE social preference
TS2-neo	Bader et al., 2011	NE spatial learning ↓ reversal learning NE initial social preference (three-chamber test) ↓ sustained social preference (three-chamber test) ↓ sustained social preference (home cage assay) NE social memory NE USV during pup separation (number of calls) NE USV during pup separation (peak frequency) NE USV during pup separation (peak amplitude) ↓ USV during pup separation (call length)
TS2-neo	Kabitzke et al., 2018	NE spatial learning NE reversal learning (trend towards worse performance) NE social preference (three-chamber test) NE social recognition ↑ social proximity NE USV during pup separation (number of calls)

Table 4: Disorder-associated cognitive and social phenotypes in *Cacna1c* mouse models (adapted from Moon et al., 2018). Effect direction always refers to the comparison with control groups. ↓ = decreased. ↑ = increased. NE = no effect. ACC = anterior cingulate cortex. CNS = central nervous system. HET = heterozygous. MWM = Morris water maze. NAcc = nucleus accumbens. PFC = prefrontal cortex. USV = ultrasonic vocalization(s).

In mice, sociability is often examined with the help of the three-chamber test developed by the Crawley lab (Nadler et al., 2004), where mice can choose between compartments containing either an unfamiliar mouse or an inanimate object. Unimpaired mice tend to prefer the compartment with the other mouse (Silverman et al., 2010). In contrast to human findings, *Cacna1c* mouse results on social interaction are mixed. On the one hand, impaired sociability was observed in two independent studies of mice with conditional developmental *Cacna1c* deletions in the PFC (Dedic et al., 2018; Kabir et al., 2017a), but not in the Nucleus accumbens (NAcc) (Terrillion et al., 2017b). Conversely, constitutive HET *Cacna1c* mice showed normal social approach behavior in one study (Dedic et al., 2018), and a sustained preference for the social stimulus in a newly developed home cage assay (Bader et al., 2011). Increased time spent in close proximity to another mouse was furthermore found in TS2-neo knock-in mice (Kabitzke et al., 2018). On the other hand, however, the same model displayed significantly reduced social preference in a prolonged version of the three-chamber test (Bader et al., 2011).

For rodents, as for humans, social communication is highly relevant to survival, as it may convey information about dangerous predators and food locations and can be used to stabilize and improve social interactions within the group (Ferretti & Papaleo, 2019). Deficits in human speech and language are more difficult to model than social interaction, though, as mice do not use a "language" per se. They do, however, communicate on other levels, such as via olfactory marking or USV (Ferretti & Papaleo, 2019; Wöhr & Scattoni, 2013). USV have so far only been investigated in TS2-neo mice and were found in one study to not differ from those emitted by WT mice between postnatal days (PND) 4-15 (Kabitzke et al., 2018), yet significantly altered in length in another pup separation paradigm (Bader et al., 2011). Olfactory abilities, on the other hand, do not appear to be affected in HET knockout mice (Dao et al., 2010).

Even more complex social behaviors, such as empathy and prosocial behavior, can be modeled using mice (Holmes, 2019; Mogil, 2019). Like humans, rodents are able to perceive and react to the emotional states of their conspecifics. This has led to the development of the observational fear learning paradigm: Some mice can learn conditioned fear from other mice without ever having experienced the aversive stimulus themselves, indicating that they are aware that the other mouse is experiencing discomfort or pain, a phenomenon that is enhanced by closer familiarity and relatedness between animals (Ferretti & Papaleo, 2019). When employing this paradigm in mice with a *Cacna1c* deletion specific to the ACC, distinctly reduced observational fear learning was discovered compared to WT mice (Jeon et al., 2010), even though regular fear conditioning was unaffected, which shows that the deficit seems to be specific to the social component of the test.

Taking evidence from both cognitive and social paradigms into account, the findings from *Cacna1c* mouse studies, while promising, once again emphasize the complexity of the relationship between the gene and disorder-relevant behavioral phenotypes. Intriguingly, the fact that the timing of genetic manipulation appears to make a significant difference to the test results (Dedic et al., 2018) is highly suggestive of the notion that further elements may be involved in the development of cognitive and social impairments associated with mental disorders.

Environmental influence

The high heterogeneity of results from clinical and preclinical studies indicates that more factors are at play in the etiology of neuropsychiatric endophenotypes than mere differences in the genome. As mentioned before, genetic factors like SNPs only ever explain a small portion of the risk (Delude, 2015; Geschwind & Flint, 2015), and even combined, the common and rare risk variants cannot explain why e.g. monozygotic twins not necessarily develop the same disorder, as shown by various heritability studies (Ronald & Hoekstra, 2011; Sullivan et al., 2003). This suggests that additional factors like environmental risks and protective influences need to be considered.

Even though data acquisition of environmental influences is difficult in human subjects, as there is no technology that can screen for the abundance of an individual's experiences (Giusti-Rodríguez & Sullivan, 2013), there are several established risk and protective factors that are assumed to contribute to disease development.

Risk & protective factors

Among these environmental influences, early life stress ranks highest in terms of prevalence and impact on future mood disorders. In fact, childhood trauma not only affects MDD diagnosis, but also determines remission rate, age of onset and general chronicity (Saveanu & Nemeroff, 2012). While inconsistent results have been obtained in bipolar research on parenting histories and early maltreatment, the methodologically stronger studies also support their association with BPD risk (Alloy et al., 2005; Rowland & Marwaha, 2018). Epidemiological risk factors for SCZ, on the other hand, include urban upbringing, a history of immigration and perinatal infections (Giusti-Rodríguez & Sullivan, 2013; Meyer-Lindenberg & Tost, 2012), although research has yielded conflicting results on the latter (Berrettini, 2005). In general, known psychosocial risk factors for disorder development later in life include social isolation, unemployment and relationship stressors (Kendler et al., 1999; Mullins & Lewis, 2017), as well as recent stressful occurrences, such as a suicide in a person's social network or sudden disability (Alloy et al., 2005; Rowland & Marwaha, 2018).

Next to overall disease development, genetic factors alone also do not fully account for the specific behavioral impairments observed in various disorders (Millan et al., 2012). Therefore, environmental factors most likely also affect cognition and social functioning. These are of relevance for not only for diagnosed patients, but for their relatives and healthy subjects alike. For example, chronic stress has been associated with WM deficits and the inability to store information in long-term memory (Bavley et al., 2017; Millan et al., 2012), and an urban upbringing may predispose an individual to weakened coping abilities which, in turn, might exacerbate social function deficits (Meyer-Lindenberg & Tost, 2012). Generally, a low socio-economic status and associated circumstances such as a lack of educational attainment are important predictors of cognitive measures later in life (Short & Baram, 2019). The most prominent negative effects, however, are linked to aversive experiences in early life.

Childhood represents a critical window of time where the developing brain is particularly sensitive for environmental insults (Sachser et al., 2018; Sandi & Haller, 2015; Short & Baram, 2019). In fact, in several neuropsychiatric disorders, the first symptoms occur during adolescence (Burrows et al., 2011). Aversive experiences during development encompass, but are not limited to verbal, physical and sexual abuse, as well as household dysfunction and stress associated with parental divorce or domestic violence (Brinker & Cheruvu, 2017). As such, early trauma has been shown to affect intermediate phenotypes, for example aggressive and hostile tendencies (Aas et al., 2016), cognitive functioning (Schenkel et al., 2005), as well as brain anatomy and activation patterns e.g. during social cue processing (Krautheim et al., 2018; van Harmelen et al., 2013). Childhood maltreatment is known to affect health and behavior far into adulthood (Cattane et al., 2018; Saveanu & Nemeroff, 2012) and can lead to altered sociability (Holmes, 2019) or promote cognitive deficits like impaired impulse control and self-regulation (Bastos et al., 2020). Early life stress can furthermore cause difficulties in recognizing facial expressions (Aas et al., 2016), negative cognitive bias (Davis et al., 2014) and impairments of spatial learning and recognition memory (Millan et al., 2012; Short & Baram, 2019).

Conversely, beneficial environmental circumstances like parental bonding, social support and positive life-events can mitigate the risk of disorder development (Alloy et al., 2005; Brinker & Cheruvu, 2017; Kircher et al., 2018; Zdun-Ryżewska et al., 2018). Among these, the most robust findings have been obtained in social support studies. The help and encouragement individuals receive from their social network can reflect positively on the disorder course (Alloy et al., 2005), symptom severity (Kendler et al., 2005) and quality of life (Zdun-Ryżewska et al., 2018). Regarding stressful life events, social and emotional support can act as a powerful protection that buffers the adverse consequences of threatening life events (TLE) and trauma in general (Brinker & Cheruvu, 2017). In fact, social support in combination with dispositional optimism is considered to be one of the most protective combinations against mental disorders (Strohmaier et al., 2013). Like risk, resilience-associated

influences such as positive caregiver experiences have also proven to be time-sensitive (Kaufman et al., 2006; Saveanu & Nemeroff, 2012), marking the phase of childhood once again as a crucial time window for potential interventions.

Gene x environment

Environmental factors appear to influence neuropsychiatric disorders and associated intermediate phenotypes significantly. In the past, a first hypothesis for their interplay with genes was the Two-Hit model, which assumed that phenotypes between individuals differed due to potential secondary environmental insults, in addition to genetic vulnerabilities (Chaste & Leboyer, 2012; Cirillo & Seidman, 2003; Schenkel et al., 2005). However, not all individuals are equally affected by risk-associated influences (Caspi & Moffitt, 2006; Saveanu & Nemeroff, 2012) and indeed, protective environments have been shown to ameliorate or even override the negative genetic effects on behavior in rodent experiments (Würbel, 2001). Multiple studies now indicate that the effect of genes and environment on disorder development may not simply be of an additive, but rather of an interactive nature (Kendler & Eaves, 1986). In fact, GxE interactions are thought to account for a large portion of unexplained phenotypic variance in genetic studies (Bastos et al., 2020; Zhao et al., 2019).

Various experimental approaches in humans, as well as rodents have already obtained evidence that GxE might also play a role in how *CACNA1C* affects behavior relevant to MDD, BPD, SCZ and ASD. In constitutive HET knockout mice, for instance, no initial difference in the reaction to chronic unpredictable stress (CUS) was discovered in terms of reduced working memory, heightened anxiety or depressive phenotype. However, several days after the CUS had subsided, *Cacna1c* HET mice exhibited a resilient phenotype compared to the control group, indicating that *Cacna1c* modulates the persistent effects of chronic stress (Bavley et al., 2017). With regard to chronic social defeat, an increased susceptibility to environmental stressors was observed in mice with reduced $Ca_v1.2$ levels in the NAcc (Terrillion et al., 2017b). The virally induced protein level reduction alone did not alter sociability, but in combination with subthreshold social defeat, impaired social interactions were revealed. Similar findings on social deficits were obtained in HET mice and those with conditional *Cacna1c* knockout in excitatory forebrain neurons during development (Dedic et al., 2018). Interestingly, mice that received the *Cacna1c* knockout during adulthood displayed a resilient phenotype regarding social defeat-induced anxiety and locomotor inhibition yet remained impaired in a spatial object recognition paradigm. More importantly, the same authors also examined human participants and found an increased risk for depressive symptoms in individuals that carried a *CACNA1C* risk allele and had experienced adverse life events in the past, theorizing that risk SNPs only predicted MDD development in those subjects with a history of trauma (Dedic et al., 2018). In another study using human participants, both *CACNA1C* genotype and exposure to TLEs were independently

associated with MDD risk, but in minor allele carriers, the risk conferred by TLEs was notably exacerbated (Zhao et al., 2019). Furthermore, A-allele carriers of a different risk SNP on *CACNA1C* intron 3 were found to be more likely to develop BPD than non-risk allele carriers if exposed to childhood trauma, indicative of a differential response to environmental stressors (Bastos et al., 2020), a notion that is in accordance with a recent study that found yet another *CACNA1C* risk allele to be associated with the development of post-traumatic stress disorder in a small cohort of traumatized police officers (Krzyzewska et al., 2018). In addition to this, another *CACNA1C* variant was among the reported top signals from three meta-analyses in a large GWAS for systematic G×E interactions in MDD (van der Auwera et al., 2018). Apart from these general disorder associations, altered ingroup and outgroup processing in the ventral ACC were observed in subjects with the combined risk of minor rs1006737 expression and childhood trauma, indicating that the risk allele interacts with environmental factors to influence brain function, as well as behavior. In the same study, these participants also exhibited higher schizotypal traits, independent of differences in the ACC (Krautheim et al., 2018).

Together, the findings from studies on humans and mice highlight how the elucidation of the way genes and environment interact to confer disorder risk during critical periods of development is vital to further understanding and eventual development of effective early interventions in at-risk populations (Marín, 2016).

Rat model & paradigms

Due to their high rate of reproduction and the ease with which their genome can be manipulated, rodent models generally provide great means to investigate behavioral and neurobiological effects of genetic manipulations (Burrows & Hannan, 2016). As described above, most studies on *Cacna1c* so far have focused on mice. In this species, however, genetic background is a known source of variability, which is why Sittig and colleagues examined several mouse strains in the context of *Cacna1c* research. They discovered significant genotype x strain interactions in phenotypic variance and concluded that most findings in *Cacna1c* knockouts are not generalizable across mouse strains (Sittig et al., 2016). This high variability in past studies reveals a pressing need for within- as well as cross-species validation studies in models with a higher translational value (Moon et al., 2018).

Luckily, advanced genetic methods have paved the way for sophisticated genome manipulations in rats (Ellenbroek & Youn, 2016; Geurts et al., 2009) – like the zinc-finger technology used for the genetic manipulation in Study I–IV of this dissertation. As the homozygous deletion of *Cacna1c* results in embryonic lethality (Seisenberger et al., 2000), a previously established method was used to generate

HET rats on a Sprague-Dawley background (Geurts et al., 2009). The rats in Study I-IV carry a four base pair deletion at 460649-460652 bp in genomic sequence resulting in an early stop codon in exon 6. This was sufficient to cause a ~50% reduction of global $Ca_V1.2$ protein levels in the brain, as described in more detail in Study I.

Interestingly, even though they are easier to handle, offer a rich spectrum of behaviors, are very intelligent and gregarious animals (Homberg et al., 2017), and thus provide certain advantages as a model species for the investigation of social behavior and cognition, *Cacna1c* studies on rats are still sparse.

Social behavior in rats

The social repertoire of rats is rich and complex, owing to their tendency to live in small borrows as large groups, characterized by distinct social hierarchies (Ellenbroek & Youn, 2016). While mice do exhibit certain social behaviors like social exploration, parenting behaviors and communal nesting, social memory and motivation for social contact (Ferretti & Papaleo, 2019), rats usually experience social interactions as more rewarding (Ellenbroek & Youn, 2016). In rats, stronger evidence has also been obtained regarding complex social behaviors like empathy (Church, 1959; Mogil, 2019) and cooperation (Rutte & Taborsky, 2007). In respect to social deficits in disorders like ASD, studies in rats are seen as especially relevant to human behavior (Ellenbroek & Youn, 2016).

In most of the mouse experiments mentioned up to this point, social behavior is commonly measured by paradigms that utilize general social preference in the three-chamber social approach test (Bader et al., 2011; Dedic et al., 2018; Kabir et al., 2017a; Kabitzke et al., 2018; Terrillion et al., 2017b). Most notably, though, while this test can also be performed using rats, their social behavior is distinguished by their prominent display of rough-and-tumble play during juvenility and their use of complex USV, which in mice are mostly observed in reproductive contexts only (Ferretti & Papaleo, 2019).

Rough-and-tumble play

From a very young age, rats being to interact frequently with their conspecifics, and high levels of rough-and-tumble play are commonly observed in juvenile animals, peaking around PND 30-40 (Panksepp, 1981; Thor & Holloway, 1984). Compare to the quantitative sociability measures in the three-chamber assay, social play is very complex and requires coordinated behaviors and communication between play partners. Several distinct moves and playful attacks can be distinguished, like pinning, wrestling and chasing (Poole & Fish, 1976). As many of these individual behaviors are relevant to adult fights between rats, social signals are essential to keep the interaction from escalating into serious altercations (Burke et al., 2017b; Kisko et al., 2017). Play behavior is an activity engaged in voluntarily and is positively reinforced via dopamine (DA) signaling in the brain's

reward system (Trezza et al., 2010), with pinning behavior typically being associated with the greatest reward (Vanderschuren et al., 2016). After short-term social deprivation, play behavior is usually increased, indicative of a heightened motivation to engage in social contact (Panksepp & Beatty, 1980). As the exposure to social stimuli is necessary to develop the ability to correctly interpret and respond to social cues (Sachser et al., 2018), play behavior in the post-weaning phase teaches young rats about appropriate behavior in social interactions during a critical period of neurobiological development (Beatty et al., 1981; Bell et al., 2010; Pellis & Pellis, 1998). Prohibition of play during juvenility, on the other hand, is known to cause significant social deficits in adult animals (Burke et al., 2017a; Hol et al., 1999).

Ultrasonic vocalizations

Next to play behavior, their complex use of USV sets rats apart. Acoustic communication is an important component of rodent social behavior, and there is a wide range of species emitting USV (Wöhr & Scattoni, 2013). Originally discovered in 1954 (Anderson, 1954), USV are used by rats in a variety of social- and non-social situations (Brudzynski, 2013; Wöhr & Schwarting, 2013). In very early life stages while they are still with their mother, rat pups produce calls in the 40-kHz range when isolated, normally eliciting maternal search and retrieval behavior (Wöhr & Schwarting, 2008). Following weaning, 40-kHz calls stop and instead a distinction emerges between 22-kHz and 50-kHz USV, which are specific to negatively and positively associated situations, respectively.

22-kHz USV are usually emitted in aversive contexts like predator exposure (Blanchard et al., 1991), social defeat (Kroes et al., 2007) and general distress (Borta et al., 2006; Knutson et al., 2002). They have been shown to lead to higher activity levels in brain regions implicated in anxiety (Demaestri et al., 2019), and in paradigms utilizing foot shocks, the emission of 22-kHz calls is highly correlated with freezing behavior (Wöhr & Schwarting, 2008). In addition, there are indications that 22-kHz USV serve as alarm calls between rats, as they have been shown to evoke behavioral inhibition in response (Fendt et al., 2018; Wöhr & Schwarting, 2007).

By contrast, 50-kHz calls rather pertain to appetitive situations, such as mating (Sewell, 1967), rough-and-tumble play (Himmler et al., 2014; Kisko et al., 2015b; Lukas & Wöhr, 2015), and the mimicking of the latter via tickling by a human experimenter (Burgdorf & Panksepp, 2001; Schwarting et al., 2007). 50-kHz calls have been described as “rat laughter”, as they are believed to reflect a positive affective state in the sender (Brudzynski, 2013; Panksepp & Burgdorf, 2003). They can also be observed in the anticipation of social play and during short-term isolation, indicating the receptiveness for social contact, and to reduce the likelihood of a potentially hostile interaction (Brenes et al., 2016; Burgdorf et al., 2008; Knutson et al., 1998). Furthermore, 50-kHz calls are heavily associated with reward,

appearing even in rewarding non-social situations (Pereira et al., 2014; Thompson et al., 2006; Wöhr & Schwarting, 2008), and being sought-after by rats, as established in studies using specifically-bred high and low 50-kHz USV "emitters" (Panksepp et al., 2002), as well as in operant conditioning tasks (Burgdorf et al., 2008). Alongside drug-induced hyperlocomotion, psychostimulants like amphetamine (AMPH) increase 50-kHz calling (Brudzynski et al., 2011; Burgdorf et al., 2001; Engelhardt et al., 2017). Moreover, the electric stimulation of mesolimbic reward pathways has been shown to elicit 50-kHz calls (Burgdorf et al., 2000), and out of all types of USV, only calls in this particular 50-kHz range stimulate dopamine release in the NAcc in both the sender (Burgdorf et al., 2007) and the receiver (Willuhn et al., 2014).

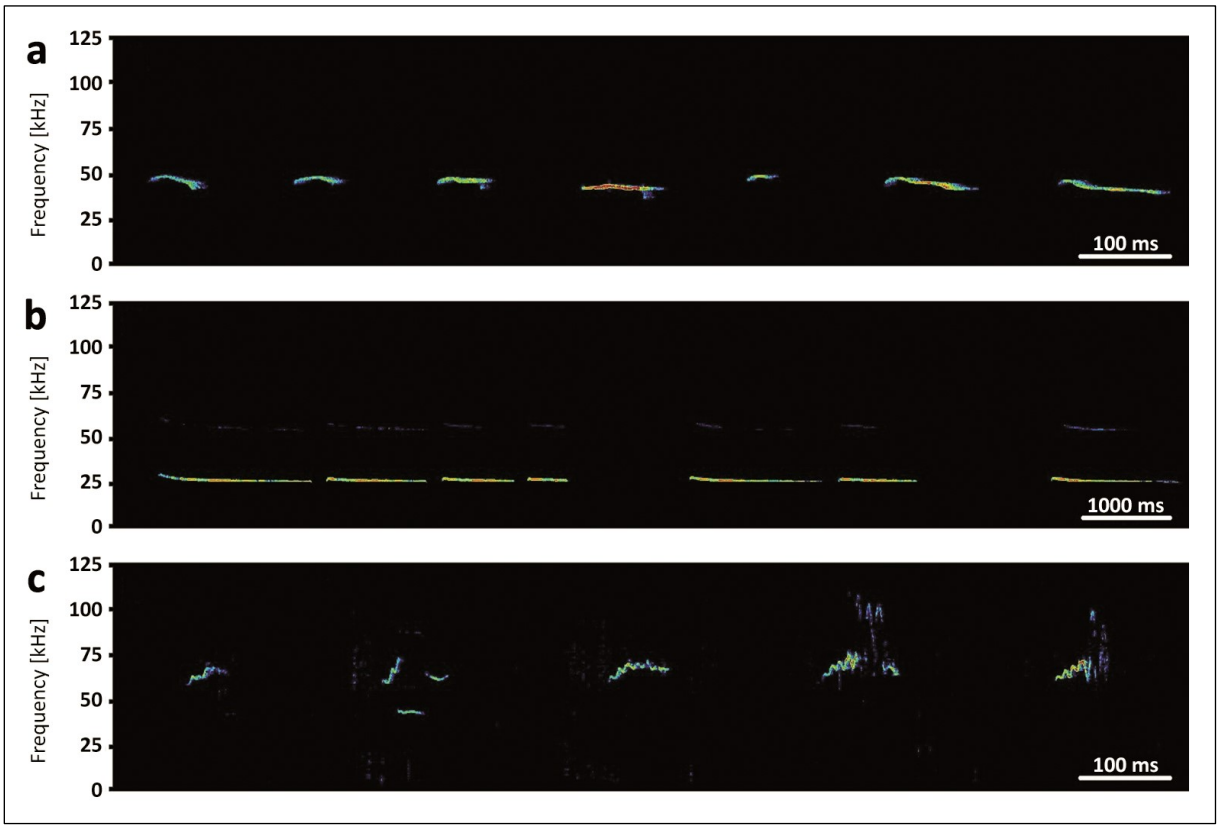

Figure 1: Spectrograms of the different types of rat USV. (a) Example of 40-kHz USV, as emitted by rat pups at separation from mother and littermates. (b) Example of 22-kHz USV, as emitted in aversive situations e.g. involving pain or exposure to predators. (c) Example of 50-kHz USV, as emitted in appetitive situations like rough-and-tumble play or mating (adapted from Wöhr, 2018).

Notably, USV production is closely linked to the display of rough-and-tumble play. In fact, various subtypes of 50-kHz calls have already been associated with specific phases and behavioral components of social play (Burke et al., 2017c; Himmler et al., 2014; Kisko et al., 2020). Specifically, 50-kHz calls are theorized to aid in keeping interactions friendly (Kisko et al., 2017). Overall, this category of vocalizations appears to be essential for normal play and social interaction in general, as suggested by evidence from studies using animals selectively bred for high vs. low calling rates (Burgdorf et al., 2013;

Panksepp & Burgdorf, 2000) and experiments with either deaf or devocalized rats (Kisko et al., 2015b; Siviy & Panksepp, 1987). Reduced 50-kHz USV emission has furthermore been linked to altered play behavior (Kisko et al., 2015a; Webber et al., 2012).

In addition, the communicative function of USV between these animals has been highlighted in several experiments utilizing playback of USV sound files without the presence of an actual rat. Presenting previously recorded 50-kHz USV to a rat reliably results in exploration and approach towards the sound source, suggesting that they function as social contact calls (Wöhr & Schwarting, 2007, 2009, 2012). These reactions are selective to 50-kHz calls and are not seen in response to background noise or time- and amplitude-matched white noise (Seffer et al., 2014; Willadsen et al., 2014). It has been proposed that the degree of exploratory and approach behavior exhibited in response to 50-kHz calls can be used as a readout for the incentive salience associated with social contact (Engelhardt et al., 2018). By comparison, the playback of 22-kHz calls recorded in aversive situations usually induces behavioral inhibition and freezing (Fendt et al., 2018; Wöhr & Schwarting, 2007), but only if the listener has previously been exposed to an aversive context themselves and learned to connect it to their own 22-kHz calls (Parsana et al., 2012). Of note, rats have been noted to sometimes respond to the playback of 50-kHz calls with 22-kHz vocalizations (Berg et al., 2018; Wöhr & Schwarting, 2009). This has been interpreted as an expression of frustration arising from the inability to locate the source of these pro-social calls (Coffey et al., 2013; Wöhr & Schwarting, 2009).

Like in social play, the approach behavior observed in response to social contact calls is particularly strong in juvenile animals (Wöhr & Schwarting, 2007) and can be used to measure the motivation for social contact (Engelhardt et al., 2017), which is of vital importance for the deficits in social behavior seen in mental disorders (Kennedy & Adolphs, 2012; Meyer-Lindenberg & Tost, 2012). Remarkably, in a recent study on a genetic rodent model for ASD, rats showed intact sociability in the three-chamber test, but significantly altered interactions in dyadic play, as well as impairments in social approach behavior to 50-kHz USV calls (Berg et al., 2018), providing evidence that these paradigms are indeed well suited to detect disease-relevant deficits.

Cognition in rats

Rats are very sensitive to rewards (Ellenbroek & Youn, 2016) and display very efficient learning strategies in a variety of paradigms aimed at cognitive function. Whereas mice have a steeper forgetting curve (Young et al., 2009), the learning performance in rats is known to be very stable and in need of fewer training sessions to reach the success criterion (Ellenbroek & Youn, 2016). Moreover, it is believed that the cognitive abilities in rats more closely mirror episodic memory in humans than those of mice (Morellini, 2013; Panoz-Brown et al., 2016). Over the last decade, rats have been

receiving more and more attention in cognition studies using genetic manipulations (Szpirer, 2020; Young et al., 2009).

Regarding research on cognitive function, a very limited number of studies has so far investigated the effect of *Cacna1c* in rats. However, there is evidence that constitutive *Cacna1c* haploinsufficiency may lead to perseverative tendencies and impaired reversal learning (Sykes et al., 2019). Calcium channels have furthermore been implicated in alterations of behavior and neurobiological structures relevant to memory (Moon et al., 2018), as well as to emotional learning in rats (Moon et al., 2020; Sykes et al., 2018). Thus far, however, no studies have examined spatial learning and relearning, or novel object recognition in *Cacna1c* rat models, despite the fact that these abilities are of particular relevance to neuropsychiatric disorders (Dere et al., 2010; Olton & Paras, 1979; Young et al., 2009), and relevant paradigms offer a range of opportunities for application in translational research.

Radial arm maze

Next to the escape-motivated water-based MWM, a popular test of spatial learning is the radial arm maze (RAM) which was originally described in 1987 (Olton, 1987) and exploits the natural requirements for rats to use spatial navigation in the search for food or shelter in different habitats (Leggio et al., 2005). Usually, the elevated maze consists of a central platform with eight long horizontal extensions (arms) arranged in a circle around the platform. At the end of each arm, there is a cup in which a food reward may be placed. Rats are normally habituated to the maze and specific food rewards in advance.

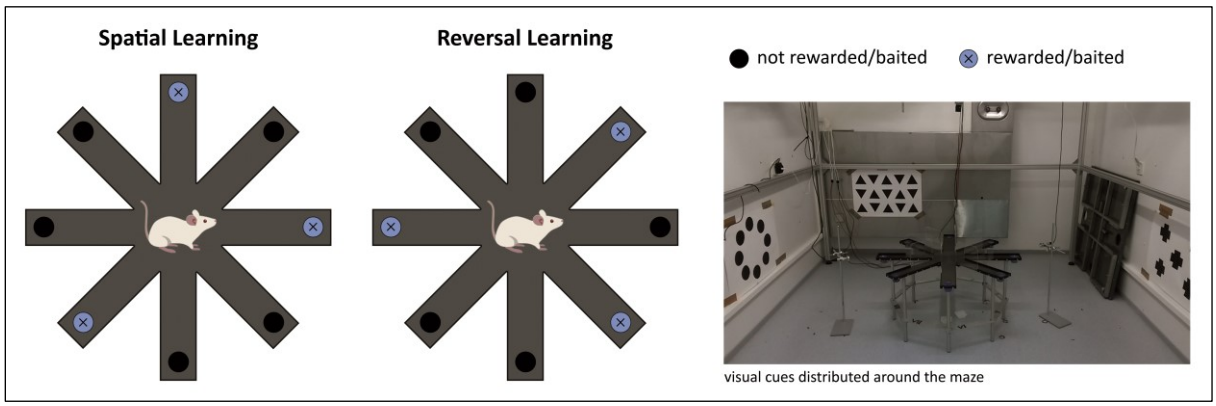

Figure 2: Radial arm maze set-up. During the phase of spatial learning (left), three arms are consistently baited. After seven consecutive days, the bait locations are switched to three different arms for the phase of reversal learning (middle). To aid orientation, visual cues are placed around the maze (right).

During spatial learning, only certain arms of the maze are baited (see Figure 2), and erroneous entries into non-baited arms are scored according to their memory requirements (Görisch & Schwarting, 2006). Entries into non-baited arms are *reference* memory (RM) errors, whereas re-entries into baited arms where rewards have already been collected are *working* memory (WM) errors. Rats can also

commit errors of a third category by re-entering non-baited arms within the same trial (*mixed* errors). To aid orientation, visual cues are typically placed around the maze for animals to navigate by. Trials are conducted over several days and are terminated after the animal has collected all rewards or reached a time criterion of e.g. five minutes.

Reversal learning can easily be examined in the RAM by switching bait locations to different arms, thereby changing the reward contingencies. In the trials following the switch, animals need to acquire and update bait locations and can only be successful if they inhibit their previously learnt behavior (Quan et al., 2010), as tendencies to persevere in arm choices result in a greater number of errors. Next to general spatial navigation abilities, optimal performance on the maze requires rats to remember bait locations, as well as their previous actions on the maze, creating a big advantage of the RAM paradigm in its simultaneous assessment of between-trial spatial reference memory and within-trial spatial working memory (Görisch & Schwarting, 2006). As spatial memory has been postulated to mirror demands in episodic memory paradigms (Morellini, 2013; Pooters et al., 2015), RAM test results can be of great translational value.

Novel object recognition

The novel object recognition test (NOR), introduced to the scientific community in 1988, is based on the rat's natural curiosity and spontaneous preference for novelty (Ennaceur & Delacour, 1988).

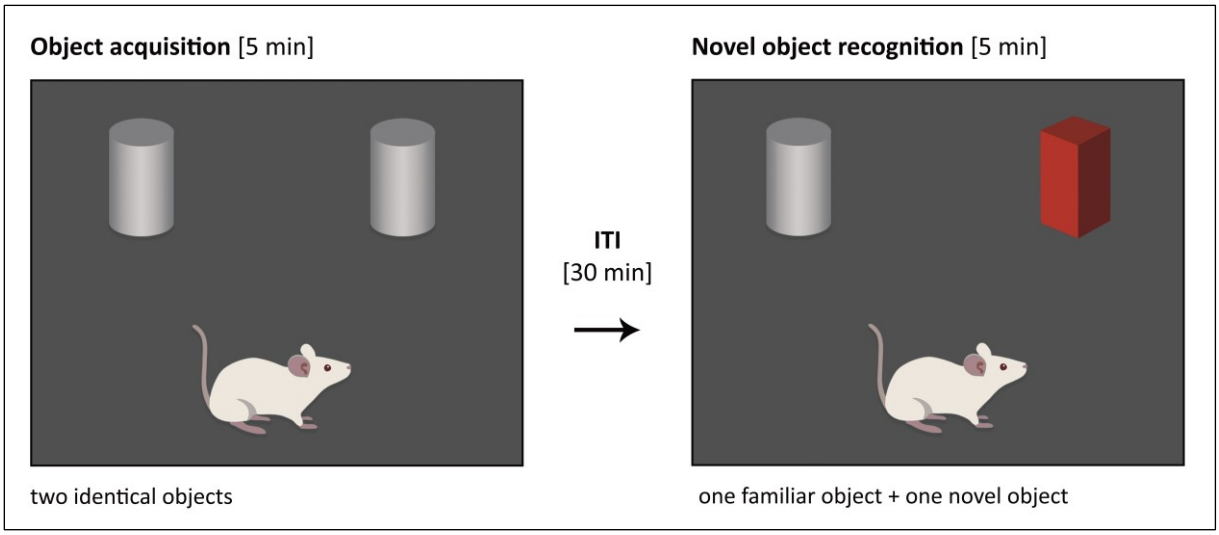

Figure 3: Novel object recognition set-up. In the first trial (left), rats are allowed to explore two identical objects for five minutes. After a delay of 30 minutes spent in their home cage, they are exposed to one familiar and one novel object for five minutes in trial 2 (right). ITI = inter-trial interval.

The standard version of this task is comprised of two trials (see Figure 3). In the first trial, an animal is exposed to two identical objects, which it can explore for a certain amount of time. After a delay, in which the rat is placed back in its home cage and the testing arena is thoroughly cleaned, the animal

is re-exposed to one of the previous objects coupled with a novel item. In this second trial, the time spent interacting with the novel object compared to the familiar object is scored. An exploration bias towards the novel object is seen as an indication that the rat recognizes the familiar object and therefore displays an increased interest in the novel one (Bevins & Besheer, 2006). As the NOR harnesses an innate instinct, it requires no previous training and is often used in translational research as a context-independent, non-spatial measure of episodic memory (Fone & Porkess, 2008).

Together, the RAM and NOR provide two adaptable and etiologically valid paradigms to examine episodic memory-like capabilities in rodents (Ennaceur & Delacour, 1988; Morellini, 2013; Olton, 1987).

Environmental manipulation

It is a popular approach to utilize genetically altered rodents under exposure to negative or positive environmental influences with the aim of exacerbating or ameliorating a certain phenotype, respectively (Burrows et al., 2011). The investigation of environmental factors in humans comes with certain disadvantages, from the enormous number of experiences and potential influences in an individual's life to the limitations imposed by the need to work with retrospective data, which can be affected by forgetting, revisionist recall or bias due to current mood or cognitive dysfunction (Zhao et al., 2019). Thus, animal models are crucial to unravel GxE interactions in disorder etiology.

As in humans, the period of juvenility is essential for the development of cognitive and social skills in the rat (Opendak et al., 2017). Indeed, it has been demonstrated that the deprivation of social contacts and social play during this crucial time results in significant social interaction and communication deficits later in life, as well as increased levels of anxiety and aggression (Burke et al., 2017a; Seffer et al., 2015), an effect that is not seen after social deprivation during adulthood (Fone & Porkess, 2008). As a matter of fact, these social deficits have only been shown to be reversible to a certain degree, if interventions like re-socialization are timed just right (Burke et al., 2017a; Lapiz et al., 2003; Seffer et al., 2015). In this context, the exposure to experimental housing conditions provides a well-suited means to introduce different environmental influences into the life of a young rat post-weaning.

Housing conditions

Various measures have already been applied to operationalize environmental conditions in animal studies (Burrows et al., 2011). Among these, the manipulation of rearing and housing conditions have a long history of yielding strong and stable effects on rodent behavior (Burrows & Hannan, 2016; Turner & Burne, 2013). With the opportunity to control both social, as well as inanimate stimulation, housing conditions offer an ideal means to control for important influences on behavioral and brain

development (Schrijver et al., 2002). As a model of maltreatment and beneficial environment, post-weaning social isolation (ISO) and social and physical enrichment (ENR), respectively, have proven to be effective in rodent models of neuropsychiatric disorders, inducing alterations in various behavioral measures including cognitive and social performance.

The social environment of an individual is essential for mental health and wellbeing and early loss of close social bonds, like parents or siblings, as well as social isolation, in general, is known to increase the risk of developing neuropsychiatric disorders in humans (Gilman et al., 2015; Meyer-Lindenberg & Tost, 2012). In rodents, post-weaning social isolation is an often-used environmental manipulation that attempts to mirror neglect and isolation during childhood. After being separated from the mother on PND 21, young rats are not placed into group housing, which is standard practice in most labs, but rather into separate cages. Here, they usually still have access to visual, auditory and olfactory stimuli provided by their conspecifics housed in the same room, but no means of direct interaction (Lapiz et al., 2003).

ISO is known to induce alterations in several behaviors with relevance to mental disorders, e.g. deficits in sucrose consumption and immobility during the forced-swim test, sensorimotor gating, increased anxiety and deviant reward response, concomitant with increased self-administration of psychostimulants (Fone & Porkess, 2008). Regarding social behavior, timing is of the essence, as brief periods of ISO increase the motivation for social contact, yet prolonged deprivation of play during juvenility leads to prominent social deficits in adulthood (Burke et al., 2017a). Post-weaning ISO furthermore increases aggression (Wongwitdecha & Marsden, 1996) yet renders the isolated animal unable to cope adequately with aggressive conspecifics (van den Berg et al., 1999). Social behaviors in adult interactions are altered and reduced (Hol et al., 1999), as are the production of and response to 50-kHz calls (Inagaki et al., 2013; Seffer et al., 2015). The negative effects on social communication are attributed to the lack of experience in social interactions, as exposure to social stimuli and direct social interaction during adolescence is essential to be able to utilize these means of communication in adulthood (Burke et al., 2017a; Sachser et al., 2018). In addition, post-weaning ISO also impairs behaviors associated with empathy, such as observational fear learning (Ferretti & Papaleo, 2019; Yusufishaq & Rosenkranz, 2013).

On the cognitive side, altered behavior in spatial learning paradigms like the MWM has been observed in several studies (Quan et al., 2010; Schrijver et al., 2002; Vorhees & Williams, 2014; Wongwitdecha & Marsden, 1996). ISO appears to have a consistent negative impact on recognition abilities in the NOR (Bianchi et al., 2006; McIntosh et al., 2013; Valluy et al., 2015), an effect that can even be induced if isolation occurs later in the life of the animal (Fischer et al., 2012). Rearing in isolation is also known to

impact behavior linked to cognitive flexibility like fear extinction, reversal learning or attentional and behavioral set-shifting (Quan et al., 2010; Schrijver et al., 2004) and tendencies towards perseveration have been found, potentially due to ISO-induced disruption of inhibitory control (Würbel, 2001). Besides these behavioral outcomes, ISO has been related to abnormalities in brain structures associated with learning memory, such as the PFC and hippocampus (Fone & Porkess, 2008; Schrijver et al., 2002). By and large, social isolation is seen as an appropriate operationalization of social isolation in humans, as it produces long-term changes in behavior and neuroanatomy of translational relevance to various neuropsychiatric disorders.

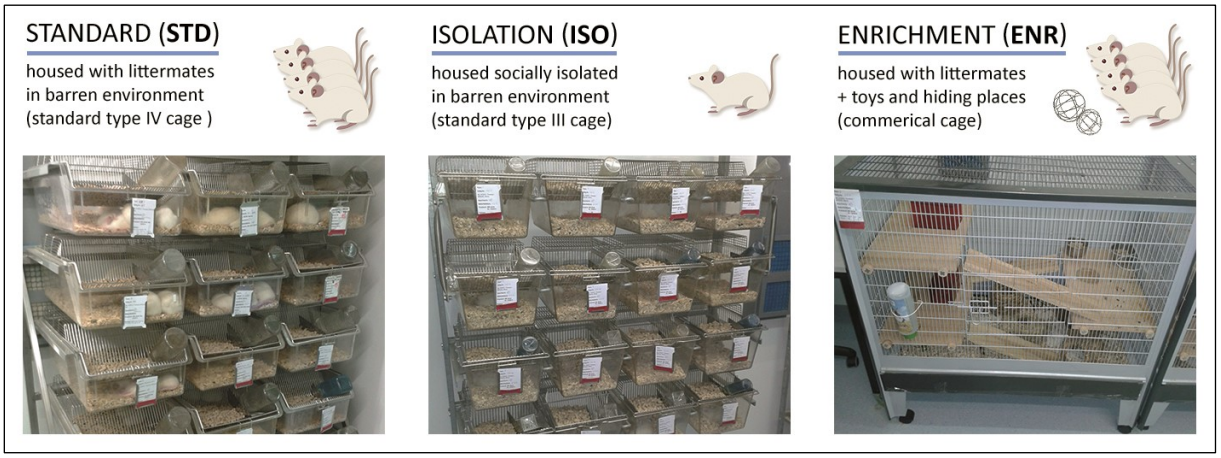

Figure 4: Experimental housing conditions, as implemented in Study IV. In the STD (left) and ENR (right) condition, rats are housed with their littermates for four weeks post-weaning, with additional physical enrichment in ENR. In ISO condition (middle), they are housed alone.

In direct contrast to the predominantly negative effects brought about by social isolation, the majority of studies on ENR has revealed positive effects. In humans, social support can reflect positively on disorder risk (Kendler et al., 2005), as well as its course (Alloy et al., 2005), and as a rule, the quality and stability of their social relationship predicts health and emotional adjustment to stress in most individuals (Kendler et al., 2005). In rodents, the importance of a complex environment was first observed by Hebb, who noticed that the rats he kept as pets at home exhibited superior problem-solving skills compared to those living in the lab (Simpson & Kelly, 2011). ENR protocols vary between labs, but generally involve the presence of more complex social and physical stimuli in an animal's immediate environment compared to standard group housing or isolation in barren cages. The inanimate objects placed in the usually larger cages can range from grass houses over swings, ropes and ladders to chew toys and tunnels. By frequently exchanging these physical components, one can increase the sensory exposure and evoke enhanced exploratory and foraging behavior in rats (Burrows et al., 2011). Both physical and social aspects of ENR are relevant, as humans, too, experience physical and social features of their environment (Short & Baram, 2019).

A number of positive ENR-induced behavioral changes have been demonstrated over the past decades. These include reduced anxiety and improved stress-coping behaviors, less depressive-like behavior (Brenes et al., 2008), as well as resilience towards the addictive properties of drugs like cocaine and AMPH and – most relevant for the focus of this thesis – augmented learning and memory capabilities (Nithianantharajah & Hannan, 2006; Simpson & Kelly, 2011). ENR rats usually display better spatial reference memory (Nilsson et al., 1999; Schrijver et al., 2004; van Praag et al., 2000), fewer working memory errors on the RAM (Leggio et al., 2005) and improved object recognition abilities (Bruel-Jungerman et al., 2005). Next to spatial learning, cognitive flexibility is also augmented in rats reared in complex environments (Garthe et al., 2016). Especially in the long term, enriched rats show superior performance in the MWM and NOR, as assessed in remote probe trials (Hullinger et al., 2015). Finally, memory-associated structures like the HC and associated synaptic plasticity rates also benefit from ENR (Nilsson et al., 1999; Würbel, 2001).

On top of these cognitive aspects, positive effects due to ENR have also been shown in terms of social behavior, where ENR causes faster emotional adaptation to novel situations (Larsson et al., 2002), as well as increased social grooming and other forms of social interaction (Burrows et al., 2011). Social ENR, in particular, has been shown to contribute to enhanced production of prosocial 50-kHz USV and social approach (Brenes et al., 2016). Together, ENR promotes positive changes in several behavioral domains and is known to have lasting effects, even when rats are returned to standard housing afterwards (Simpson & Kelly, 2011).

Objectives & hypotheses

This dissertation strives to shed light on how the *CACNA1C* gene interacts with environmental factors to influence intermediate phenotypes related to neuropsychiatric disorders. To this aim, we used a constitutive HET *Cacna1c* knockout rat model in combination with environmental manipulation as an additional hit or protective factor and embarked on a large effort to phenotype these animals, focusing on social and cognitive behaviors in Study I-IV with several unpublished data yet to come.

In **Study I** we explored the role of *Cacna1c* in regulating socio-affective communication after weaning during the critical developmental period of adolescence in rats. The exact role of *Cacna1c* in social impairments is not clear, and inconsistent result patterns have been observed in mouse studies (Bader et al., 2011; Dedic et al., 2018; Kabir et al., 2017a; Kabitzke et al., 2018). As complex social behaviors can be investigated in a more sophisticated manner in rats with their rich behavioral repertoire (Berg et al., 2018; Ellenbroek & Youn, 2016), we expect that the examination of juvenile USV production in

combination with rough-and-tumble play will reveal social deficits in our male *Cacna1c* haploinsufficient animals, as compared to their WT siblings.

In **Study II**, we aimed at further exploring the role of *Cacna1c* in regulating behavioral phenotypes, focusing on sex-specific differences in social behavior and communication during the post-weaning phase. Sex differences have previously been reported in *Cacna1c* findings (Dao et al., 2010; Zanos et al., 2015), as well as in studies examining social play and the emission of 50-kHz calls during play, and in playback paradigms (Argue & McCarthy, 2015; Himmler et al., 2014; Wöhr, 2018). We hope to be able to replicate the findings from the male rats of Study I in females and anticipate prominent deficits in the play behavior and pro-social USV production of our female *Cacna1c* rats.

Next to social impairments, cognitive dysfunction is highly relevant for neuropsychiatric disorders and considered to be an important predictor of functional outcomes and recovery rates (Green et al., 2000; MacQueen & Memedovich, 2017; Millan et al., 2012). **Study III** therefore set out to investigate cognitive functioning in our newly developed genetic *Cacna1c* rat model. Mouse models with genetic manipulation of *Cacna1c* expression levels have mostly found cognitive deficits (Dedic et al., 2018; Moosmang et al., 2005a; Temme et al., 2016; White et al., 2008), which is why we expect our animals to show an equally impaired learning and memory performance.

As noted above, environmental influences are strongly suspected to interact with genetic factors to influence, as well as to directly affect disorder-associated cognitive phenotypes (Bianchi et al., 2006; Leggio et al., 2005; Quan et al., 2010). Because of this, our goal in **Study IV** was to observe the long-term interactive effects of diverse environmental housing manipulations in a critical developmental period with *Cacna1c* haploinsufficiency on cognitive measures in adulthood. Based on previous findings on experimental housing, we expect ISO to exhibit a negative influence on cognition and ENR to impact learning positively.

Publications

Summary of publications

Study I: Kisko et al., 2018

Kisko, T. M., Braun, M. D.*, Michels, S., Witt, S. H., Rietschel, M., Culmsee, C., Schwarting, R. K. W., & Wöhr, M. (2018). Cacna1c haploinsufficiency leads to pro-social 50-kHz ultrasonic communication deficits in rats. Disease Models & Mechanisms, 11(6).*

Journal Impact Factor: 4.028

The cross-disorder risk gene *CACNA1C* is strongly implicated in multiple neuropsychiatric disorders, including autism spectrum disorder, bipolar disorder and schizophrenia, with deficits in social functioning being common for all major neuropsychiatric disorders. To examine the gene's role in social impairments, we compared WT Sprague-Dawley males to male rats haploinsufficient for the *Cacna1c* gene, as generated by means of zinc-finger technology, in assays of social interaction and communication. Namely, animals were exposed to 50-kHz USV playback on a radial maze and same-genotype pairs were allowed to engage in rough-and-tumble play on three consecutive days during adolescence following short-term isolation. During social play, 50-kHz USV emission was recorded. Furthermore, we controlled for repetitive and stereotyped patterns of behavior by measuring self-grooming and predilection to circling. Our results show that a deletion of *Cacna1c* leads to deficits in social behavior and pro-social 50-kHz USV in rats. Reduced levels of 50-kHz USV 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 calls in the sender, *Cacna1c* deletion reduced social approach behavior elicited by playback of 50-kHz USV. 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.

* shared first authorship

Study II: Kisko et al., 2020

Kisko, T. M., Braun, M. D.*, Michels, S., Witt, S. H., Rietschel, M., Culmsee, C., Schwarting, R. K. W., & Wöhr, M. (2018). Sex-dependent effects of Cacna1c haploinsufficiency on juvenile social play behavior and pro-social 50-kHz ultrasonic communication in rats. Genes, Brain, and Behavior, 19(2), e12552.*

Journal Impact Factor: 3.157

There is evidence for sex-dependent influences of single-nucleotide polymorphisms within *CACNA1C* on diagnosis, course, and recovery in humans. To investigate how the gene influences social behavior in a sex-dependent manner, we compared rough-and-tumble play, emission of pro-social 50-kHz USV, and social approach behavior in response to playback of 50-kHz USV between constitutive HET females and WT littermate controls, and contrasted these findings to data previously reported in males. To ensure deviations from normal social behavior were not due to deficits in olfactory perception, we also employed the olfactory habituation and dishabituation paradigm. The results from this study show for the first time that partial depletion of *Cacna1c* leads to sex-dependent deviation in social behavior and communication in rats. In females, *Cacna1c* haploinsufficiency led to hypermasculinization, with rough-and-tumble play behavior, in general, and pinning behavior, in particular, being even higher than in males without affecting concomitant 50-kHz calls. In males, by contrast, rough-and-tumble play behavior was not altered, yet emission of 50-kHz USV was diminished in constitutive HET animals. The behavioral responses elicited by playback of 50-kHz USV were reduced upon partial *Cacna1c* depletion in both sexes. It thus can be concluded that *Cacna1c* plays a prominent sex-dependent role in regulating juvenile rat social play behavior and pro-social 50-kHz ultrasonic communication with relevance to sex-specific effects seen in neuropsychiatric disorders.

* shared first authorship

Study III: Braun et al., 2018

Braun, M. D., Kisko, T. M.*, Vecchia, D. D., Andreatini, R., Schwarting, R. K. W., & Wöhr, M. (2018). Sex-specific effects of Cacna1c haploinsufficiency on object recognition, spatial memory, and reversal learning capabilities in rats. Neurobiology of Learning and Memory, 155, 543–555.*

Journal Impact Factor: 3.010

Because *CACNA1C* is strongly implicated in mental disorders characterized by deficits in cognitive functioning, our goal was to use a newly developed HET *Cacna1c* rat model to examine the gene's role in cognition. Specifically, this study set out to investigate spatial and reversal learning on the radial arm maze, as well as object recognition memory in the NOR paradigm in HET rats in comparison to WT littermate controls of both sexes. Our results show that both WT and HET animals were able to learn the rewarded arm configuration of a radial maze over the course of seven 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 HET females showing hypoactivity and fewer mixed memory errors. In males, a difference was found during probe trials for both learning phases, with HET rats displaying better distinction between previously baited and non-baited arms, and regarding cognitive flexibility in favor of the WT animals. All experimental groups proved to be sensitive to reward magnitude and fully able to distinguish between novel and familiar items in the NOR task. Taken together, these results indicate that while HET animals display initial impairments in cognitive flexibility and females appear hypoactive at the start of learning, *Cacna1c* haploinsufficiency has a minor, but positive impact on long-term spatial learning in rats and does not affect their ability to distinguish novel objects from familiar items in either sex.

* shared first authorship

Study IV: Braun et al., 2019

Braun, M. D., Kisko, T. M.*, Witt, S. H., Rietschel, M., Schwarting, R. K. W., & Wöhr, M. (2019). Long-term environmental impact on object recognition, spatial memory and reversal learning capabilities in Cacna1c-haploinsufficient rats. Human Molecular Genetics, 28(24), 4113–4131*

Journal Impact Factor: 4.544

Genetic and environmental influences are thought to interact in their contribution to the etiology of neuropsychiatric disorders like MDD, BPD, SCZ and ASD. Here, we used our constitutive HET *Cacna1c* rat model in combination with a 4-week exposure to either post-weaning ISO, standard housing or social and physical ENR during the critical juvenile developmental period between PND 21 and PND 50. Subsequently, animals were subjected to the NOR paradigm on PND 90-93, as well as to food reward-motivated spatial and reversal learning on the radial arm maze on PND 120-130. The results of this study provide evidence for a gene × environment interaction, i.e. an interplay between *Cacna1c* haploinsufficiency and environment during juvenile development, on object recognition, spatial memory and reversal learning capabilities in adulthood. ENR had a positive influence on HET rats and WT littermate controls in spatial and reversal learning, while post-weaning social isolation negatively affected novel object recognition in both genotypes. Despite intact spatial learning and re-learning abilities in all groups, slight but consistent long-term deficits were evident in HET rats previously housed under standard conditions, particularly during reversal learning, that were not apparent in HET animals previously exposed to ENR. Together, this supports the notion that *Cacna1c* interacts with the environment to shape disease vulnerability and associated long-term alterations in cognitive functioning.

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RESEARCH ARTICLE

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

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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.

This article has an associated First Person interview with the first author of the paper.

KEY WORDS: $Ca_v1.2$, Calcium, Autism, Social behavior, Rough-and-tumble play, Ultrasonic vocalizations

INTRODUCTION

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. $Ca_v1.2$ accounts 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 et al., 2011). Importantly, rats begin interacting socially at a 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 (Schneider and Koch, 2005) manipulations.

Acoustic communication is another important component of their social behavior repertoire. Rats emit whistle-like calls in the ultrasonic range, i.e. ultrasonic vocalizations (USVs) (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 USVs 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., 2015). 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 USVs can be used as a behavioral readout for the incentive salience of social contact (Engelhardt et al., 2017).

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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 USVs, including both sender and receiver. Effects of *Cacna1c* haploinsufficiency were assessed in male constitutive heterozygous *Cacna1c*$^{+/-}$ rats (N=20) and compared to wild-type *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 in *Cacna1c*$^{+/-}$ rats are reduced by slightly more than 50% in the brain, as compared to *Cacna1c*$^{+/+}$ littermates (t_{10}=4.345, P=0.001; Fig. 1; for representative western blot and antibody validation, see Fig. S1).

Rough-and-tumble play and pro-social 50-kHz USVs

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

Despite unchanged rough-and-tumble play behavior, however, *Cacna1c*$^{+/-}$ rats emitted fewer 50-kHz USVs than *Cacna1c*$^{+/+}$ littermates while engaged in playful encounters (G: $F_{1,17}$=7.708, P=0.013; Fig. 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: $F_{1,17}$=1.537, P=0.232). Irrespective of genotype, there was an increase in 50-kHz USV emission during anticipation (D: $F_{2,35}$=8.498, P=0.001; D×G: $F_{2,35}$=1.057, P=0.359) and during playful social interactions (D: $F_{2,35}$=20.901, P<0.001; D×G: $F_{2,35}$=0.025, P=0.976) across play sessions.

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; Fig. 2D). Within play periods, 50-kHz USV levels differed between individual rough-and-tumble play components [component (C): $F_{2,36}$=16.159, P<0.001] and genotypes specifically during wrestling, with *Cacna1c*$^{+/-}$ rats emitting fewer 50-kHz USVs than *Cacna1c*$^{+/+}$ littermates (t_{18}=2.529, P=0.021; Fig. 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: Fig. 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 versus wrestling: t_{19}=3.783, P=0.004; pinning versus chasing: t_{19}=4.529, P=0.001; wrestling versus chasing: t_{19}=0.438, P=0.672), and during chasing but not pinning and wrestling in *Cacna1c*$^{+/-}$ rats (pinning versus wrestling: t_{19}=2.124, P=0.063; pinning versus chasing: t_{19}=3.737, P=0.005; wrestling versus chasing: t_{19}=2.370, P=0.042).

Moreover, differences in the prevalence of specific 50-kHz USV subtypes was evident [subtype (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 USVs [criteria previously established by Pereira et al. (2014) and repeatedly applied by Engelhardt et al. (2017) and Wöhr et al. (2015); Fig. 3A] 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 USVs were not affected by genotype (t_{18}=1.650, P=0.116 and t_{18}=0.295, P=0.771, respectively; Fig. 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; S×G: $F_{3,36}$=2.406, P=0.083; S×C: $F_{6,72}$=3.545, P=0.004; S×C×G: $F_{6,72}$=2.774, P=0.018; Fig. 3B; for representative ethograms: Fig. 3C). In *Cacna1c*$^{+/+}$ littermates, pinning was primarily associated with the occurrence of flat 50-kHz USVs and, to a lesser extent, mixed 50-kHz USVs, while trill and step 50-kHz USVs were rarely emitted. A similar pattern was obtained in *Cacna1c*$^{+/-}$ rats, with a large number of flat 50-kHz USVs, moderate levels of mixed and trill 50-kHz USVs, but low rates of step 50-kHz USVs. During wrestling, *Cacna1c*$^{+/+}$ littermates emitted high rates of mixed and flat 50-kHz USVs, together with moderate numbers of trill 50-kHz USVs but low numbers of step 50-kHz USVs. This was different in *Cacna1c*$^{+/-}$ rats, which produced a high number of trill and flat 50-kHz USVs during wrestling but relatively low numbers of mixed and particularly step 50-kHz USVs. During chasing, high levels of mixed 50-kHz USVs, moderate rates of flat and trill 50-kHz USVs, but low levels of step 50-kHz USVs were evident in *Cacna1c*$^{+/+}$ littermates. In *Cacna1c*$^{+/-}$ rats, trill 50-kHz USVs were most prominent, while flat, mixed and step 50-kHz USVs did not occur often during chasing. In rare cases, both rats were emitting 50-kHz USVs at the same time. The number of such overlapping 50-kHz USVs did not differ between genotypes

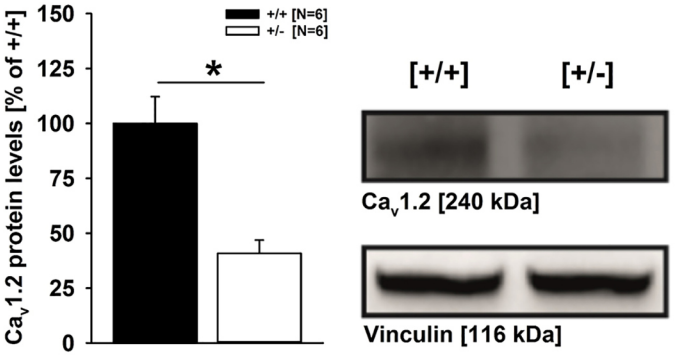

Fig. 1. $Ca_V1.2$ protein levels in *Cacna1c*$^{+/-}$ rats and *Cacna1c*$^{+/+}$ littermate controls. $Ca_V1.2$ expression levels were analyzed by western blot from cortical tissue of male *Cacna1c*$^{+/-}$ rats (white bar; N=6) and *Cacna1c*$^{+/+}$ littermate controls (black bar; N=6). The bar graph (left panel) was 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±s.e.m. *P<0.050 vs *Cacna1c*$^{+/+}$ littermate controls.

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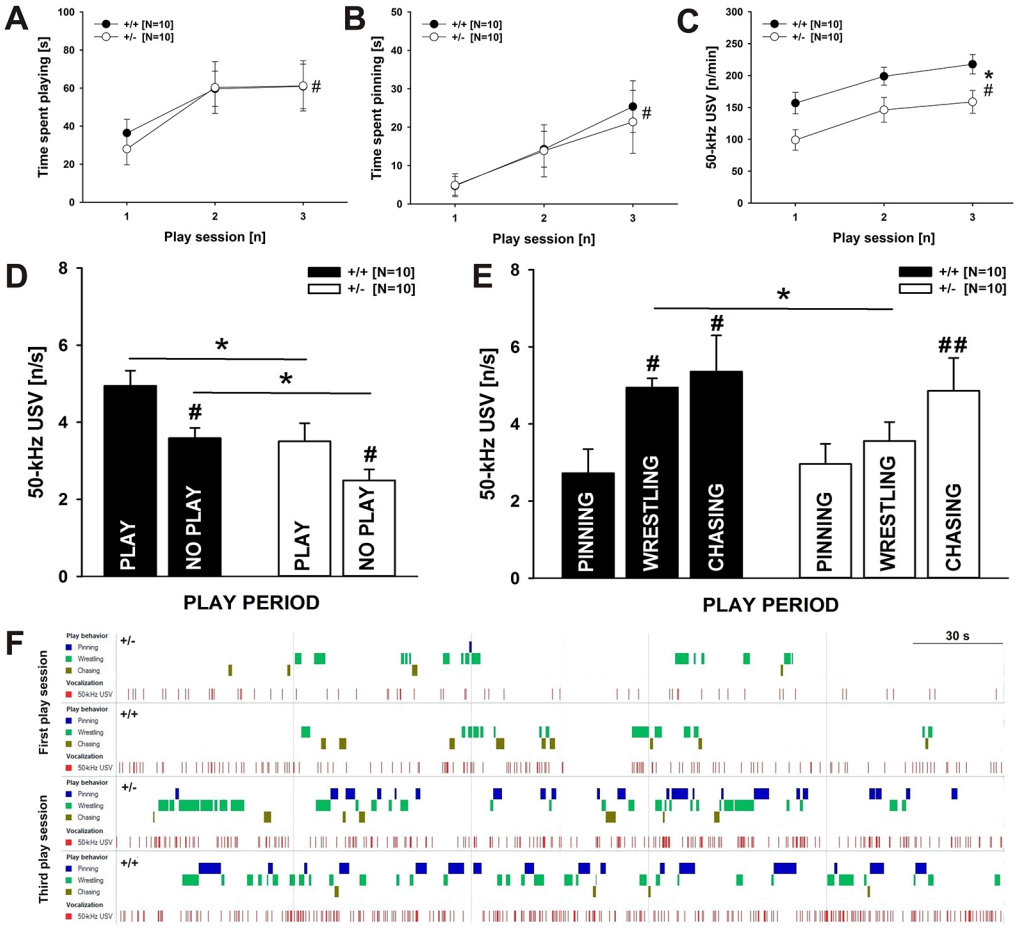

Fig. 2. Rough-and-tumble play behavior and concomitant pro-social 50-kHz USV emission in *Cacna1c*$^{+/-}$ rats and *Cacna1c*$^{+/+}$ littermate controls. (A) Time spent playing, (B) time spent pinning and (C) 50-kHz USV emission across the three play sessions in male *Cacna1c*$^{+/-}$ rats (white circles; *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 bars; *N*=10) and *Cacna1c*$^{+/+}$ littermate controls (black bars; *N*=10), with 50-kHz USVs 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 USVs (red) for the entire 5 min play sessions. Data are presented as mean±s.e.m. $^{\#}P$<0.050 vs first play session (in A-C), vs play (in D), vs pinning (in E); $^{\#\#}P$<0.050 vs pinning and wrestling (in E); $^{*}P$<0.050 vs *Cacna1c*$^{+/+}$ littermate controls.

(t_{18}=1.472, P=0.158). Occasionally, atypical 50-kHz USVs 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 USVs differed between genotypes. While call duration was not affected (t_{18}=0.987, P=0.337; Fig. 4A), 50-kHz USVs emitted by *Cacna1c*$^{+/-}$ rats were characterized by higher peak frequencies than the ones emitted by *Cacna1c*$^{+/+}$ littermates (t_{18}=2.677, P=0.015; Fig. 4B), without differing in frequency modulation (t_{18}=0.259, P=0.799; Fig. 4C). Moreover, 50-kHz USVs emitted by *Cacna1c*$^{+/-}$ rats were lower in peak amplitude (t_{18}=3.330, P=0.004; Fig. 4D). The increase in peak frequency seen in *Cacna1c*$^{+/-}$ rats was driven by a categorical shift in the relative occurrence of 50-kHz USVs within two prominent call clusters. In both genotypes, two clusters were clearly evident. In the first cluster, 50-kHz USVs are characterized by relatively low peak frequencies, between 50 and 70 kHz. In the second cluster, 50-kHz USVs are characterized by substantially higher peak frequencies, between 70 and 90 kHz. *Cacna1c*$^{+/+}$ littermates emitted more low-frequency first-cluster 50-kHz USVs than high-frequency second-cluster 50-kHz USVs. Conversely, *Cacna1c*$^{+/-}$ rats emitted about the same number of first- and second-cluster 50-kHz USVs, 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 (Fig. 4E).

Playback of pro-social 50-kHz USVs

Importantly, low emission of pro-social 50-kHz USVs in the sender was paralleled by reduced responsivity to such 50-kHz USVs in the receiver, with 50-kHz USVs but not the acoustic control stimulus white noise (Fig. 5A) leading to social approach behavior, as demonstrated by means of our established 50-kHz USV radial maze playback paradigm (Fig. 5B). Specifically, the acoustic control stimulus white noise induced behavioral inhibition [time (T): $F_{1,38}$=104.143, P<0.001; TxG: $F_{1,38}$=0.134, P=0.717; Fig. 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; T×G: $F_{1,38}$=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 USVs. 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; T×G: $F_{1,38}$=0.005, P=0.945 and T: $F_{1,38}$=0.977, P=0.329; T×G: $F_{1,38}$=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; T×G: $F_{14,532}$=1.270, P=0.222). Immediate head orientation in response to playback of 50-kHz USVs and white noise was seen in almost all rats (~95%) and did not differ between genotypes (χ^2=2.105, P=0.147). Not a single rat failed to respond to both acoustic stimuli by head orientation.

Social approach behavior in response to playback of 50-kHz USVs was reflected in a preference for arms proximal to the ultrasonic loudspeaker [T: $F_{1,38}$=50.904, P<0.001; preference (P):

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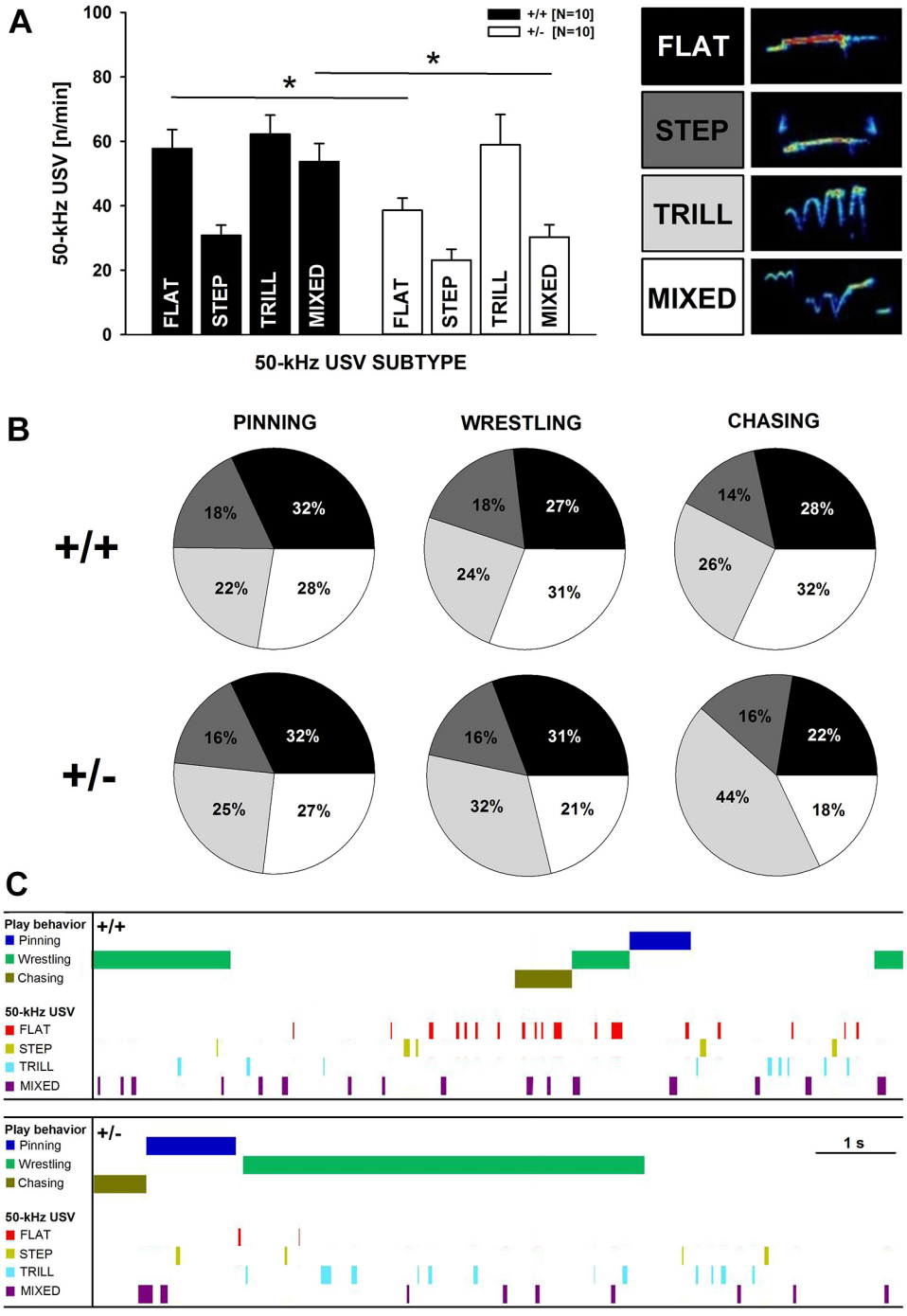

Fig. 3. Subtypes of pro-social 50-kHz USVs emitted by *Cacna1c*$^{+/-}$ rats and *Cacna1c*$^{+/+}$ littermate controls during rough-and-tumble play behavior. (A) Emission of the different 50-kHz USV subtypes, i.e. flat, step, trill and mixed 50-kHz USVs, in male *Cacna1c*$^{+/-}$ rats (white bars; *N*=10) and *Cacna1c*$^{+/+}$ littermate controls (black bars; *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, during the third play session. The proportion of flat, step, trill and mixed 50-kHz USVs 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±s.e.m. *P<0.050 vs *Cacna1c*$^{+/+}$ littermate controls.

$F_{1,38}$=68.242, P<0.001; T×P: $F_{1,38}$=103.775, P<0.001]. This preference was strongly dependent on genotype (T×G: $F_{1,38}$=0.977, P=0.329; P×G: $F_{1,38}$=1.292, P=0.263; T×P×G: $F_{1,38}$=8.015, P=0.007; Fig. 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; T×G: $F_{1,38}$=0.612, P=0.439; P: $F_{1,38}$=19.212, P<0.001; P×G: $F_{1,38}$=7.609, P=0.009; T×P: $F_{1,38}$=13.409, P=0.001; T×P×G: $F_{1,38}$=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).

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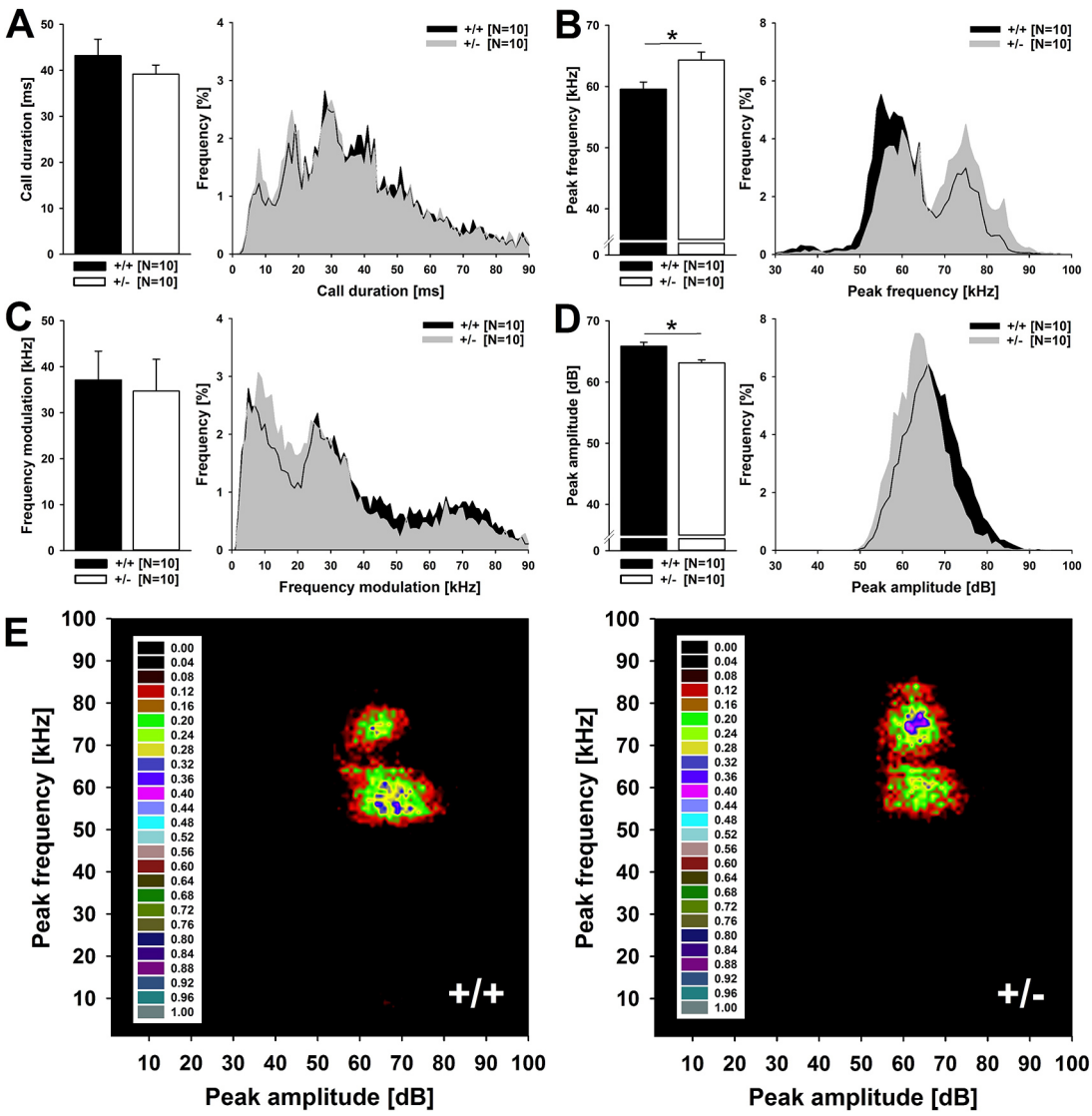

Fig. 4. Acoustic characteristics of pro-social 50-kHz USVs emitted by *Cacna1c*$^{+/-}$ rats and *Cacna1c*$^{+/+}$ littermate controls during rough-and-tumble play behavior. (A) Call duration [in milliseconds (ms)]; (B) peak frequency [in kilohertz (kHz); (C) frequency modulation (in kHz); and (D) peak amplitude [in decibel (dB)] of 50-kHz USVs emitted by male *Cacna1c*$^{+/-}$ rats (white bars and gray frequency histograms reflecting percentage of occurrence; *N*=10) and *Cacna1c*$^{+/+}$ littermate controls (black bars and black frequency histograms reflecting percentage of occurrence; *N*=10) during the third play session. (E) Density plots depicting the distribution of individual 50-kHz USVs depending on peak frequency (in kHz) and peak amplitude (in dB) emitted by male *Cacna1c*$^{+/-}$ rats (+/–; *N*=10) and *Cacna1c*$^{+/+}$ littermate controls (+/+; *N*=10) during the third play session. Color coding reflects frequencies of occurrence as percentages. Density plots were generated by including more than 8000 50-kHz USVs emitted by male *Cacna1c*$^{+/-}$ rats and more than 10,000 50-kHz USVs emitted by *Cacna1c*$^{+/+}$ littermate controls. Data are presented as mean±s.e.m. *P<0.050 vs *Cacna1c*$^{+/+}$ littermate controls.

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; T×G: $F_{1,38}$=0.085, P=0.772; P: $F_{1,38}$=5.421, P=0.025; P×G: $F_{1,38}$=11.467, P=0.002; T×P: $F_{1,38}$=4.885, P=0.033; T×P×G: $F_{1,38}$=1.289, P=0.263; Fig. 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; T×G: $F_{1,38}$=1.672, P=0.204; P: $F_{1,38}$=8.358, P=0.006; P×G: $F_{1,38}$=13.943, P=0.001; T×P: $F_{1,38}$=4.959, P=0.032; T×P×G: $F_{1,38}$=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).

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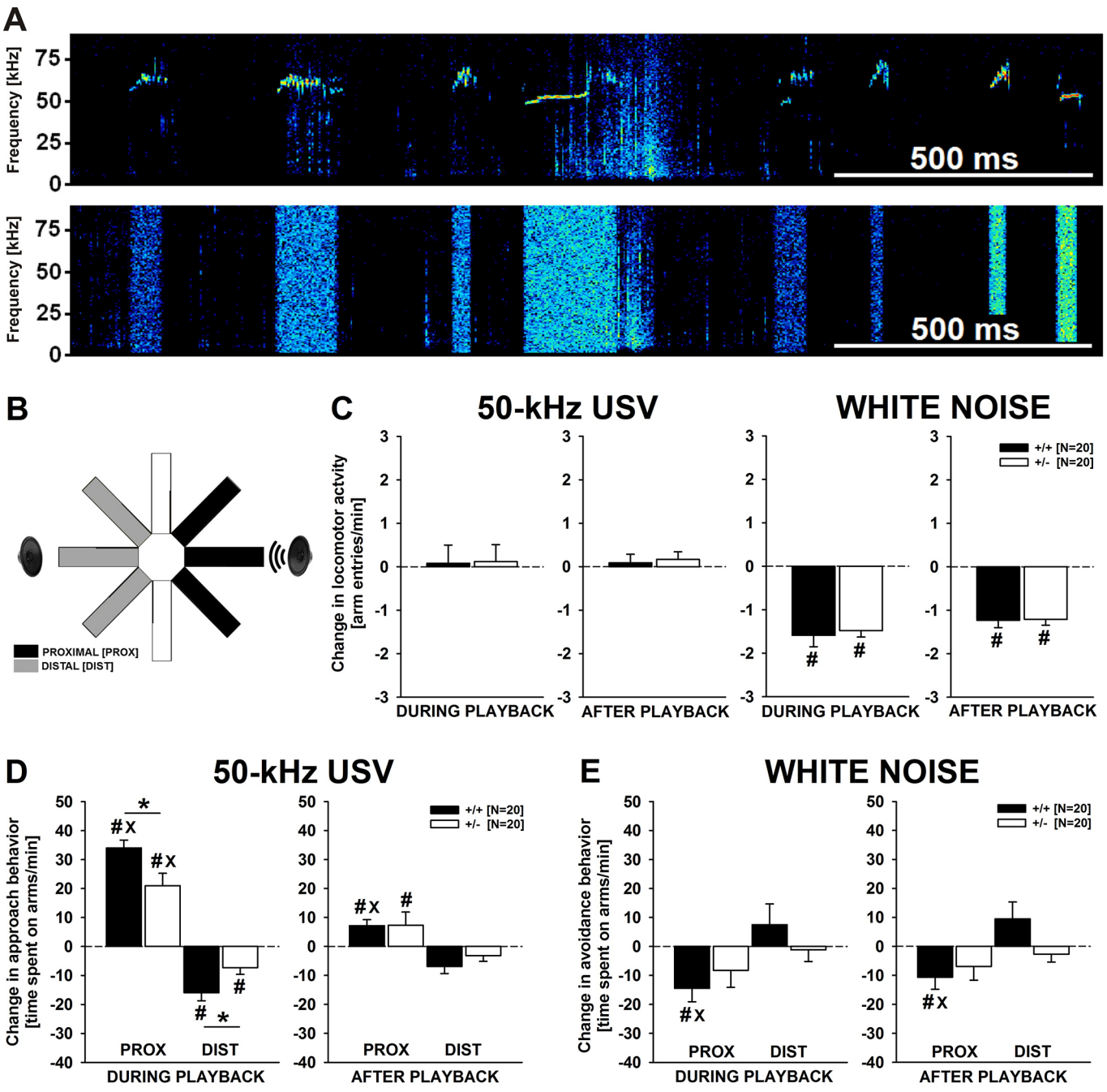

Fig. 5. Social approach behavior evoked by pro-social 50-kHz USV playback in *Cacna1c*$^{+/-}$ rats and *Cacna1c*$^{+/+}$ littermate controls. (A) Exemplary spectrograms of acoustic stimuli used for playback, namely pro-social 50-kHz USVs (upper panel) and time- and amplitude-matched white noise (lower panel). (B) Schematic illustration of the radial maze used for playback depicting proximal (black), distal (gray) 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 min 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 min 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 and distal arms per minute during (left) and after (right) white noise playback, as compared to the 5 min baseline period before playback. The dashed line represents baseline levels. Data are presented as mean±s.e.m. $^{\#}P$<0.050 vs baseline levels; $^{\times}P$<0.050 vs distal; $^{*}P$<0.050 vs *Cacna1c*$^{+/+}$ littermate controls.

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; Fig. S3A) and self-grooming (t_{38}=1.127, P=0.267; Fig. 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.

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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 USVs was strongly affected. Over all three play sessions, *Cacna1c*$^{+/-}$ rats consistently emitted fewer 50-kHz USVs 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 USVs were detected during wrestling. Within play periods, the genotype difference in 50-kHz USVs 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 USVs was enhanced in *Cacna1c*$^{+/-}$ rats at the expense of mixed 50-kHz USVs. 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 USVs 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 USVs 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 USVs 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 USVs 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 USVs are more prominent in our newly developed rat model than in a well-established *Shank3* rat model for ASD (Berg et al., 2018), 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

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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, although, in rats, *Cacna1c* haploinsufficiency already results in deficits, is possibly due to the richer social behavior repertoire of rats, with pro-social 50-kHz USVs being particularly sensitive for detecting disorder-relevant behavioral phenotypes.

In summary, reduced levels of 50-kHz USVs 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 USVs in the sender, *Cacna1c* deletion reduced the behavioral responses elicited by 50-kHz USV playback. 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.

MATERIALS AND METHODS

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).

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 wild-type *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 460,649-460,652 bp in the 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; 58×38×20 cm, length×width×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, applied using a 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 and 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: 5′-GCTGCTGAGCCTTTTATTGG-3′ (*Cacna1c* Cel-1 F) and 5′-CCTCCT-GGATAGCTGCTGAC-3′ (*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 ***g*** at 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). A total of 50 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^{TM} 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 USVs (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 USVs

On PND 32-34, rough-and-tumble play behavior and the emission of pro-social 50-kHz USVs 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 the play phase) in an, at first, unfamiliar observation arena (35×35 cm, with Plexiglas walls; floor covered with 1 cm of fresh bedding) after one rat of the pair had been habituated to the test environment for 2 min (referred to as the anticipation phase). A 3 day 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 USVs (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 USVs

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 to testing in a Makrolon type III cage (265×150×425 mm, plus high stainless-steel covers; Tecniplast Deutschland GmbH), and isolation was maintained throughout the 3-day 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 USVs 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 USVs were performed for the third play session by means of high-resolution ethograms using The Observer XT. 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 USVs were measured under red light (~28 lux).

Playback of pro-social 50-kHz USVs

On PND 24±3, social approach behavior in response to pro-social 50-kHz USVs was assessed on an elevated radial eight-arm maze (arms: 40.5×9.8 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 USVs 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: (1) pro-social 50-kHz USVs and (2) white noise; the latter serving as a time- and amplitude-matched acoustic stimulus control (Seffer et al., 2014). Pro-social 50-kHz USVs 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 USVs 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 min 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 min baseline period before playback from the time spent on proximal and distal arms per minute during and after 50-kHz USV playback. USVs were monitored with two ultrasonic condenser microphones (CM16, Avisoft Bioacoustics) placed next to the loudspeakers.

Recording and analysis of USVs

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 USVs emitted by juvenile rats during rough-and-tumble play was provided by an experienced observer, who manually counted the number of USVs in 20 s time bins. If two 50-kHz USV elements were at least 10 ms apart, two independent 50-kHz USVs were counted. Based on previous studies on 50-kHz USVs, additional parameters were determined for ~20,000 50-kHz USVs 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 USVs 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 (Engelhardt et al., 2017; Wöhr et al., 2015) criteria. Only rats emitting more than five 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 USVs with comparatively low peak frequencies below 32 kHz and/or long call durations higher than 150 ms was determined. Finally, overlapping 50-kHz USVs, i.e. when both rats were emitting 50-kHz USVs at the same time, were included in the detailed spectrographic analysis. One subject rat was excluded from the analysis of USVs of the first play session due to data loss.

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 (265×150×425 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 USVs 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 USVs 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 USVs, repetitive and stereotyped patterns of behavior, line crossings and rearing events, and $Ca_v1.2$ protein levels were compared between genotypes by means of unpaired *t*-tests. The χ^2-test was applied to compare immediate head orientation between genotypes. A *P*-value of <0.050 was considered statistically significant.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: T.M.K., M.D.B., R.K.W.S., M.W.; Methodology: T.M.K., M.D.B., R.K.W.S., M.W.; Investigation: T.M.K., M.D.B., S.M.; Resources: S.H.W., M.R., C.C., R.K.W.S.; Writing - original draft: T.M.K., M.W.; Writing - review & editing: T.M.K., M.D.B., S.M., S.H.W., M.R., C.C., R.K.W.S., M.W.; Visualization: T.M.K., S.M., M.W.; Supervision: S.H.W., C.C., M.W.; Project administration: M.W.; Funding acquisition: S.H.W., M.R., C.C., R.K.W.S., M.W.

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Supplementary information

Supplementary information available online at
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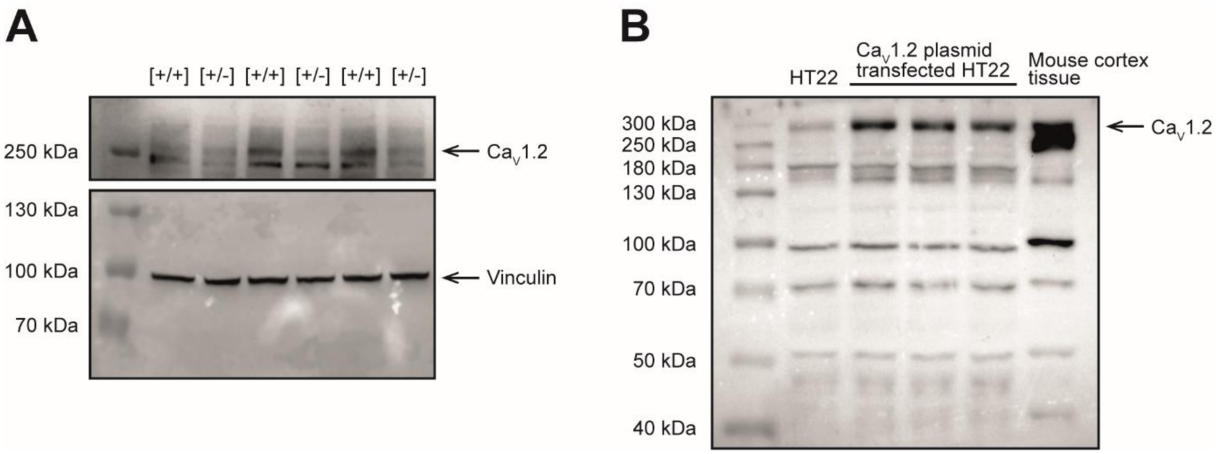
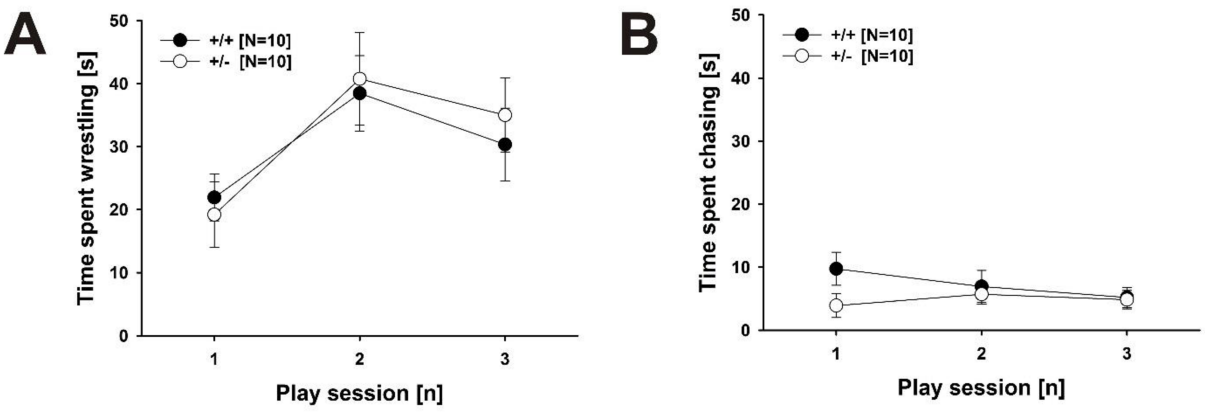
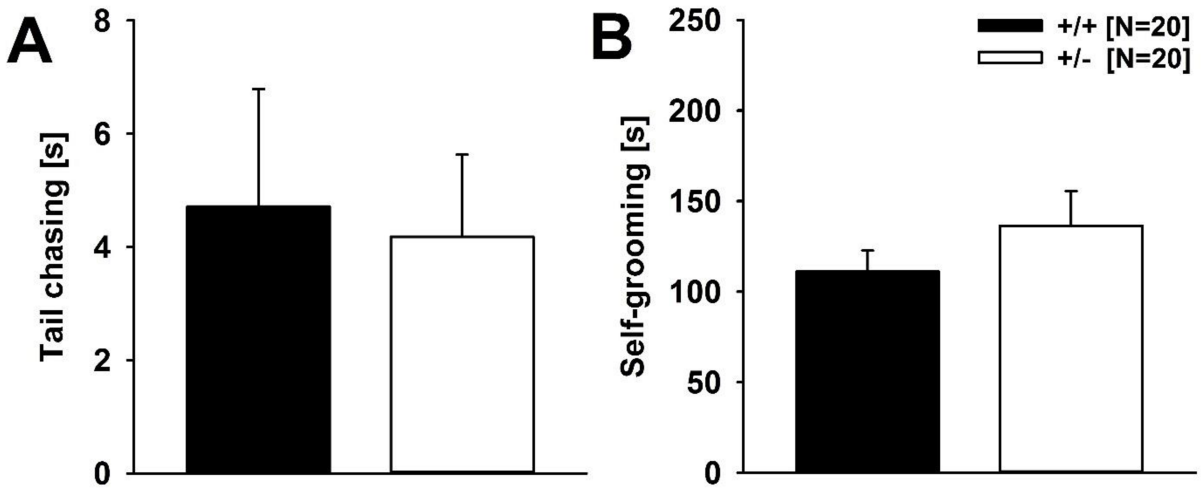


Supplementary Figure S1: Representative whole immunoblot from rat cortical tissue of *Cacna1c*$^{+/-}$ males ([+/-]) and *Cacna1c*$^{+/+}$ littermate controls ([+/+]). (**A**) The upper part of the membrane was incubated with anti-Cav1.2 antibody (1:500, Cat# ACC-003, Lot# ACC003AN5102, Alomone Labs, 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.

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ORIGINAL ARTICLE

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

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As cross-disorder risk gene, *CACNA1C* is implicated in the etiology of all major neuropsychiatric disorders characterized by deficits in social behavior and communication and there is evidence for sex-dependent influences of single-nucleotide polymorphisms within *CACNA1C* on diagnosis, course, and recovery in humans. In this study, we aimed, therefore, at further exploring the role of *Cacna1c* in regulating behavioral phenotypes, focusing on sex-specific differences in social behavior and communication during the critical developmental period of adolescence in rats. Specifically, we compared rough-and-tumble play, concomitant emission of pro-social 50-kHz ultrasonic vocalizations, and social approach behavior in response to playback of 50-kHz ultrasonic vocalizations between constitutive heterozygous *Cacna1c*$^{+/-}$ females and wildtype *Cacna1c*$^{+/+}$ littermate controls, and contrasted present female findings to data previously reported in males. Our results show for the first time that partial depletion of *Cacna1c* leads to sex-dependent alterations in social behavior and communication in rats. In females, *Cacna1c* haploinsufficiency led to hypermasculinization, with rough-and-tumble play behavior, in general, and pinning behavior, in particular, being even higher than in males without affecting concomitant 50-kHz ultrasonic vocalizations. In males, in contrast, rough-and-tumble play behavior was not altered, yet emission of 50-kHz ultrasonic vocalizations was diminished following partial *Cacna1c* depletion. The behavioral responses elicited by playback of 50-kHz ultrasonic vocalizations were reduced upon partial *Cacna1c* depletion in both sexes. It thus can be concluded that *Cacna1c* plays a prominent sex-dependent role in regulating juvenile rat social play behavior and pro-social 50-kHz ultrasonic communication with relevance to sex-specific effects seen in neuropsychiatric disorders.

KEYWORDS

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

1 | INTRODUCTION

Continued effort has revealed the highly polygenic nature of a broad range of complex neuropsychiatric disorders in humans. Many of the individual associations identified in genetic studies are shared across multiple disorders, indicating extensive pleiotropy and challenging the biological validity of current diagnostic strategies.[1–3] For instance, as

Abbreviations: ASD, autism spectrum disorder; BPD, bipolar disorder; Ca^{2+}, calcium ion; *Cacna1c*$^{+/-}$, heterozygous *Cacna1c*; *Cacna1c*$^{+/+}$, wildtype *Cacna1c*; MDD, major depressive disorder; PND, postnatal day; SCZ, schizophrenia; SD, Sprague-Dawley; SNP, single-nucleotide polymorphism; USV, ultrasonic vocalizations.

Theresa M. Kisko and Moria D. Braun have shared first authorship to this study.

cross-disorder risk gene, *CACNA1C* is implicated in the etiology of all major neuropsychiatric disorders, most notably autism spectrum disorder (ASD),[4–7] schizophrenia (SCZ),[8–11] major depressive disorder (MDD),[8,12–14] and bipolar disorder (BPD).[15–18] *CACNA1C* encodes the α1C subunit of the voltage-gated L-type calcium ion (Ca^{2+}) channel $Ca_v1.2$. With voltage sensor and conduction pore, the α1C subunit is a key regulator of membrane permeability and thus depolarization-dependent Ca^{2+} influx into the cell. $Ca_v1.2$ accounts for ~90% of all voltage-gated L-type Ca^{2+} channels in the brain and represents a promising therapeutic target.[19]

CACNA1C is ranked as a prime candidate susceptibility gene,[20–22] most notably because single-nucleotide polymorphisms (SNPs) within *CACNA1C* belong to the best replicated and most robust genetic findings from genome-wide association studies in psychiatry.[23–25] Further evidence from clinical studies links rs1006737, the primary *CACNA1C* risk allele associated with neuropsychiatric disorders, to alterations in brain structure and function, both in patients[26–29] and healthy individuals.[30–33] For instance, rs1006737 is associated with alterations in neural correlates of facial emotion recognition,[34–36] social outgroup processing,[37] and verbal fluency.[38] Interestingly, the effects of rs1006737 and other SNPs on diagnosis, associated personality traits, and relevant resilience factors were reported to be sex-dependent, with more prominent effects in women than in man.[12,39,40]

A common feature of all major neuropsychiatric disorders is reduced social functioning.[41,42] This includes impairments in direct reciprocal interaction, lack of social approach, and failure of normal back-and-forth conversation. Rats are a useful model system to study neurobiological mechanisms underlying deficits in social functioning with relevance to neuropsychiatric disorders in humans[43,44] and in a recent study we obtained evidence for social behavior and communication deficits in male *Cacna1c* haploinsufficient rats.[45] As highly gregarious animals living in large groups characterized by prominent social hierarchies, rats display a rich and complex social behavior repertoire.[46] Already from a very young age, rats begin interacting socially and engage in high levels of social play behavior[47–49] particularly during the middle of the juvenile stage,[50] including pinning, wrestling, and chasing. Typically, male rats are studied[51] because they express a higher frequency of social play and engage in rougher defense tactics than females.[52,53] Vital to healthy development, social play contributes more than just a high value of reward to the participants[54] and it is believed that playful interactions during the juvenile stage are a necessary condition for adequate acquisition of social competence and skills, like the expression and interpretation of communicative signals from conspecifics.[55]

Acoustic communication is another prominent element of the rat social behavior repertoire. In fact, in a broad range of social situations characterized by high emotional valence, rats emit whistle-like calls above the human hearing range, so-called ultrasonic vocalizations (USV).[56–58] Converging evidence from selective breeding, surgical devocalization, and sound playback studies supports the notion that such USV serve as situation-dependent socio-affective signals, with specific USV types fulfilling distinct communicative functions. Specifically, 22-kHz USV occur in aversive situations, such as the exposure to predators, fighting, and fear learning, and are thought to reflect a negative affective state of the sender. They serve an alarm function and induce behavioral inhibition in the recipient rat.[59,60] In contrast, 50-kHz USV are believed to reflect a positive affective state ("rat laughter"),[61] as they are emitted in appetitive situations, with particularly high emission rates during and in anticipation of rough-and-tumble play[62–64] or when social play is mimicked through an experienced human experimenter by tickling.[65–68] Interestingly, we recently found the emission of 50-kHz USV to be strongly affected by partial *Cacna1c* depletion.[45] Despite no alterations in rough-and-tumble play behavior, 50-kHz USV were consistently reduced across all play sessions in male *Cacna1c* haploinsufficient rats, possibly reflecting reduced *liking*[69] of playful interactions. This reduction was paralleled by alterations in the prevalence of subtype variations of 50-kHz USV,[45] including, for example, FLAT or TRILL 50-kHz USV,[62,70,71] previously associated with distinct affective states and communicative functions.[72–74]

As repeatedly demonstrated in playback studies, 50-kHz USV serve important pro-social communicative functions. Most notably, they induce social exploratory activity and approach behavior in the recipient rat, probably by eliciting the anticipation of rewarding social contact.[60,75] Like rough-and-tumble play, the social approach response is particularly prominent during the juvenile stage,[60] supporting its application as a behavioral measure for quantifying the incentive salience of social contact.[76] In line with the notion that 50-kHz USV function as social contact calls acting to (re)establish or maintain social proximity, young rats prefer to spend time with conspecifics displaying high 50-kHz USV levels[77] and the potential for social contact greatly increases their production.[78] Moreover, experimental evidence indicates that 50-kHz USV promote and maintain playful interactions.[79] If rats are unable to emit 50-kHz USV, social play behavior decreases,[79] and, as a consequence, the risk for developing severe social impairments increases, as reflected, for example, in a complete lack of social approach to 50-kHz USV.[80] In fact, in our recent study in male *Cacna1c* haploinsufficient rats,[45] we found that a decrease in $Ca_v1.2$ expression levels not only leads to impairments in ultrasonic communication in the sender but also in the recipient rat. Specifically, the reduction in 50-kHz USV emission displayed by *Cacna1c* haploinsufficient rats during social play was paralleled by reduced social approach in response to 50-kHz USV playback, suggesting less *wanting* in addition to the reduction in the *liking* component associated with rough-and-tumble play.[69]

Due to the sex-dependent influences of SNPs within *CACNA1C* on diagnosis, course, and recovery in humans,[12,39,40] we reasoned that a sex-specific assessment of social behavior and communication with relevance to neuropsychiatric disorders in *Cacna1c* haploinsufficient rats might provide novel insights into the complex role *CACNA1C* appears to play. In *Cacna1c* mouse models, evidence for sex-dependent *Cacna1c* depletion effects was provided in the context of emotion regulation and drug sensitivity as well as learning and memory. Specifically, *Cacna1c* haploinsufficiency in mice was reported to result in increased anxiety-related behavior, decrease development of learned helplessness, decreased startle responsivity, and greater attenuation of amphetamine-induced hyperlocomotion in females than in males.[12] Moreover, evidence for a sex-dependent modulation of age-related cognitive decline was obtained in mice[81] and we recently found sex-specific effects of *Cacna1c* haploinsufficiency on

spatial memory and reversal learning in rats.[82] To our knowledge, sex-specific effects of *Cacna1c* haploinsufficiency in the context of social behavior and communication have not yet been studied in relevant rodent models.

In this study, we aimed, therefore, at further exploring the role of *Cacna1c* in regulating behavioral phenotypes, focusing on sex-specific differences in social behavior and communication during the critical developmental period of adolescence in rats. To this aim, we took advantage of our previously developed genetic *Cacna1c* rat model[45] and applied behavioral assays specifically designed to truly capture the interactive and reciprocal nature of social encounters. Specifically, we compared rough-and-tumble play, concomitant emission of pro-social 50-kHz USV, and social approach behavior in response to playback of 50-kHz USV between constitutive heterozygous *Cacna1c*$^{+/-}$ females and wildtype *Cacna1c*$^{+/+}$ littermate controls, and contrasted present female findings to data previously reported in males.[45] Male and female data were obtained in the same study, including all relevant littermate controls.

2 | METHODS

2.1 | 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*$^{+/-}$ rats and *Cacna1c*$^{+/+}$ littermate controls were reported before,[45] but extended by additional analyses where necessary for the sake of sex comparison. *Cacna1c*$^{+/-}$ rats were generated by means of zinc finger technology[83] by SAGE Labs (now Horizon Discovery Ltd., Cambridge, UK) on a Sprague-Dawley (SD) background. *Cacna1c*$^{+/-}$ rats carry a 4 base pair deletion at position 460 649-460 652 in genomic sequence, resulting in an early stop codon in exon 6. Genotyping was performed using DNA obtained from tail samples on a 3130xl Genetic Analyzer (Thermo Fisher Scientific, Waltham, Massachusetts) as described before,[45] with the following primers: 5′-GCTGCTGAGCCTTTTATTGG-3′ (*Cacna1c* Cel-1 F) and 5′-CCTCCTGGATAGCTGCTGAC-3′ (*Cacna1c* Cel-1 R).

As reported before,[45] a heterozygous breeding protocol was applied to obtain *Cacna1c*$^{+/-}$ offspring and *Cacna1c*$^{+/+}$ littermate controls. Briefly, SD females (Charles River, Sulzfeld, Germany) and male *Cacna1c*$^{+/-}$ rats were paired for breeding. The day of birth was defined as postnatal day (PND) 0. 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. After weaning on PND 21, rats were socially housed in mixed-genotype groups of 4-6 with same-sex littermate partners under standard laboratory conditions. Standard rodent chow and water were available ad libitum. Rats were identified by paw tattoo. To avoid litter effects, only litters with both genotypes and sexes being present were included in the experiments.

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, Regierungspräsidium Gießen, Germany).

2.2 | Protein analysis

Protein extraction and Western blot were performed using frozen cortical tissue pieces (25-50 mg, left hemisphere) from 10 months old female *Cacna1c*$^{+/-}$ rats (N = 6) and their *Cacna1c*$^{+/+}$ littermate controls (N = 6), applying a protocol described before,[45] with the following antibodies: anti-Ca_v1.2 (1:500; Cat# ACC-003; Lot# ACC003AN5102; Alomone Labs, Jerusalem, Israel) and anti-Vinculin (1:20000; Sigma-Aldrich, München, Germany). Chemiluminescence was imaged with a ChemiDoc XRS system and protein quantification was performed using Bio-Rad Image Lab Software (Bio-Rad Laboratories, Hercules, California).

2.3 | Behavioral phenotyping

Behavioral phenotypes were studied in male and female *Cacna1c*$^{+/-}$ rats and compared to *Cacna1c*$^{+/+}$ littermate controls during the critical developmental period of adolescence by applying our established test battery,[45] consisting of the (I) 50-kHz USV radial maze playback paradigm (PND 24 ± 3), (II) rough-and-tumble play behavior and pro-social 50-kHz USV (PND 32-34), (III) as well as repetitive and stereotyped patterns of behavior (PND 64 ± 3). In addition, (IV) olfactory habituation and dishabituation was performed (PND 75 ± 3). All rats were handled for three consecutive days prior to behavioral testing in a standardized way and then tested in all four behavioral assays. Testing took place during the light phase of a 12:12 hours light/dark cycle. Behavioral analysis was performed by a trained observer blind to experimental condition.

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

On PND 32 to 34, rough-and-tumble play behavior and the emission of pro-social 50-kHz USV were measured, exactly as described before.[45] In brief, on three consecutive days, pairs of juvenile rats were allowed to socially interact for 5 minutes (referred to as play phase) in an, at first, unfamiliar observation arena after one rat of the pair being habituated to the test environment for 2 minutes (referred to as anticipation phase). Rats were always paired with a same-sex, same-genotype, age-matched nonlittermate and noncagemate partner. To enhance social motivation, rats were socially isolated for 24 hours prior testing and single housing was maintained throughout testing. The following behavioral measures were scored by a trained observer using The Observer XT (Noldus, Wagenigen, The Netherlands): (a) duration of rough-and-tumble play (including pinning, wrestling, and chasing), (b) duration of social investigation (including sniffing the anogenital and head/neck regions of the partner), and (c) duration of physical contact, with the latter two being considered as social but nonplayful behaviors.

2.5 | Playback of pro-social 50-kHz ultrasonic vocalizations

On PND 24 ± 3, social exploratory activity and approach behavior in response to pro-social 50-kHz USV was assessed on an elevated radial eight-arm maze, exactly as described before.[45] In brief, acoustic stimuli were presented through an ultrasonic loudspeaker (ScanSpeak, Avisoft Bioacoustics) placed 20 cm away from the end of one arm. Two acoustic stimuli were used: (a) pro-social 50-kHz USV and (b) White Noise; the latter serving as a time- and amplitude-matched acoustic stimulus control.[84] After an initial 15 minutes habituation period, each recipient rat was exposed to 1 minute playback presentations of 50-kHz USV and White Noise, separated by a 10 minutes interstimulus interval. Stimulus order was counterbalanced. Total number of arm entries served as locomotor activity measure. 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 relative to the 5 minutes baseline period before playback.

2.6 | Recording and analysis of ultrasonic vocalizations

As reported before,[45] UltraSoundGate Condenser CM16 Microphones connected to an UltraSoundGate 416H USB audio device (Avisoft Bioacoustics) were used for USV recordings. Briefly, acoustic data was recorded with a sampling rate of 250 000 Hz (recording range: 0-125 kHz; 16 bit) by Avisoft RECORDER USGH and transferred to Avisoft SASLab Pro for acoustical analysis. High-resolution spectrograms (488 Hz, 0.512 ms) were calculated through fast Fourier transformation (512 FFT length, 100% frame, Hamming window, 75% time window overlap). Detection of pro-social 50-kHz USV emitted during rough-and-tumble play was performed by a trained observer, who manually counted their numbers in 20 seconds time bins. Of note, aversive 22-kHz USV occurred very rarely during rough-and-tumble play and were, therefore, not included in the analysis. During playback of pro-social 50-kHz USV, however, 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.[76]

2.7 | Repetitive and stereotyped patterns of behavior

On PND 64 ± 3, repetitive and stereotyped patterns of behavior were tested in a clean cage without bedding material, exactly as described before.[45] In brief, repetitive and stereotyped patterns of behavior were assessed by measuring the duration of self-grooming and circling behavior during tail-chasing. For quantifying locomotor activity, the test cage was virtually divided by a line into two halves and the numbers of line crossings and rearing events were counted.

2.8 | Olfactory habituation and dishabituation

On PND 75 ± 3, olfactory habituation and dishabituation was tested in a clean Makrolon type III cage (265 × 150 × 425 mm, plus high stainless-steel covers; Tecniplast Deutschland GmbH) with fresh bedding material. A cage with fresh bedding was used for each 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 rat for 45 minutes to the cage, with a clean cotton-tipped wooden applicator suspended from the cage lid to be well within reach of the rat. During testing, each rat was presented with five different odors, that is, plain tap water, two nonsocial odors, and two social odors, as described previously.[85] The test consisted of 15 sequential 2 minutes trials, with three consecutive trials per odor and an intertrial interval of 1 minute: 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, Michigan), 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. Nonsocial 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 nonsocial 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 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 rat, scoring of the time spent sniffing was stopped and restarted the next trial. One rat was excluded from statistical analysis due to data loss. Olfactory habituation and dishabituation was tested once under white light (~30 lx) conditions.

2.9 | Statistical analysis

Analyses of variance (ANOVAs) for repeated measurements were used to compare rough-and-tumble play behavior and pro-social 50-kHz USV between genotypes 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, and 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, and ExSxG). Cav1.2 protein levels were compared using unpaired *t* tests. A *P*-value of <0.050 was considered statistically significant.

3 | RESULTS

In this study, we compared rough-and-tumble play, concomitant emission of pro-social 50-kHz USV, and social approach behavior in response to playback of 50-kHz USV between constitutive heterozygous *Cacna1c*$^{+/-}$ females and wildtype *Cacna1c*$^{+/+}$ littermate controls, and contrasted the female findings to data obtained in males.[45] As shown by Western blot, $Ca_V1.2$ protein levels in *Cacna1c*$^{+/-}$ females were reduced by slightly more than 50% in the brain, as compared to *Cacna1c*$^{+/+}$ littermates (t_{10} = 3.942; *P* = 0.003; Figure 1), in line with our earlier findings from male rats.[45]

3.1 | 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*$^{+/+}$ littermates (t_{18} = 3.039; *P* = 0.007). Another 4 weeks later then, on PND 64 ± 3, that is, 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, body weight did not differ between genotypes, as previously reported.[45]

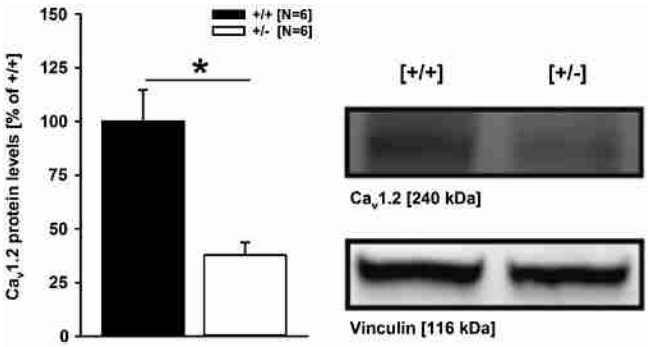

FIGURE 1 $Ca_V1.2$ protein levels in *Cacna1c*$^{+/-}$ rats and *Cacna1c*$^{+/+}$ littermate controls. $Ca_V1.2$ expression levels were analyzed by Western blot from cortical tissue of female *Cacna1c*$^{+/-}$ rats (white bars; *N* = 6) and *Cacna1c*$^{+/+}$ littermate controls (black bars; *N* = 6). 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 female *Cacna1c*$^{+/+}$ littermate controls

3.2 | 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; Supporting Information Figure S1), while chasing was not affected by genotype (G: $F_{1,18}$ = 2.343; *P* = 0.143; Figure S1B). When performing more detailed genotype comparisons across test days, the time spent 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*$^{+/+}$ littermates 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), whereas 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 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*$^{+/+}$ littermates 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

TABLE 1 Body weight in *Cacna1c*$^{+/-}$ rats and *Cacna1c*$^{+/+}$ littermate controls

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

Abbreviations: PND, postnatal day; USV, ultrasonic vocalizations.
*P < 0.050 vs *Cacna1c*$^{+/+}$ littermate controls; Male data were reported before[45] and included for the sake of comparison.

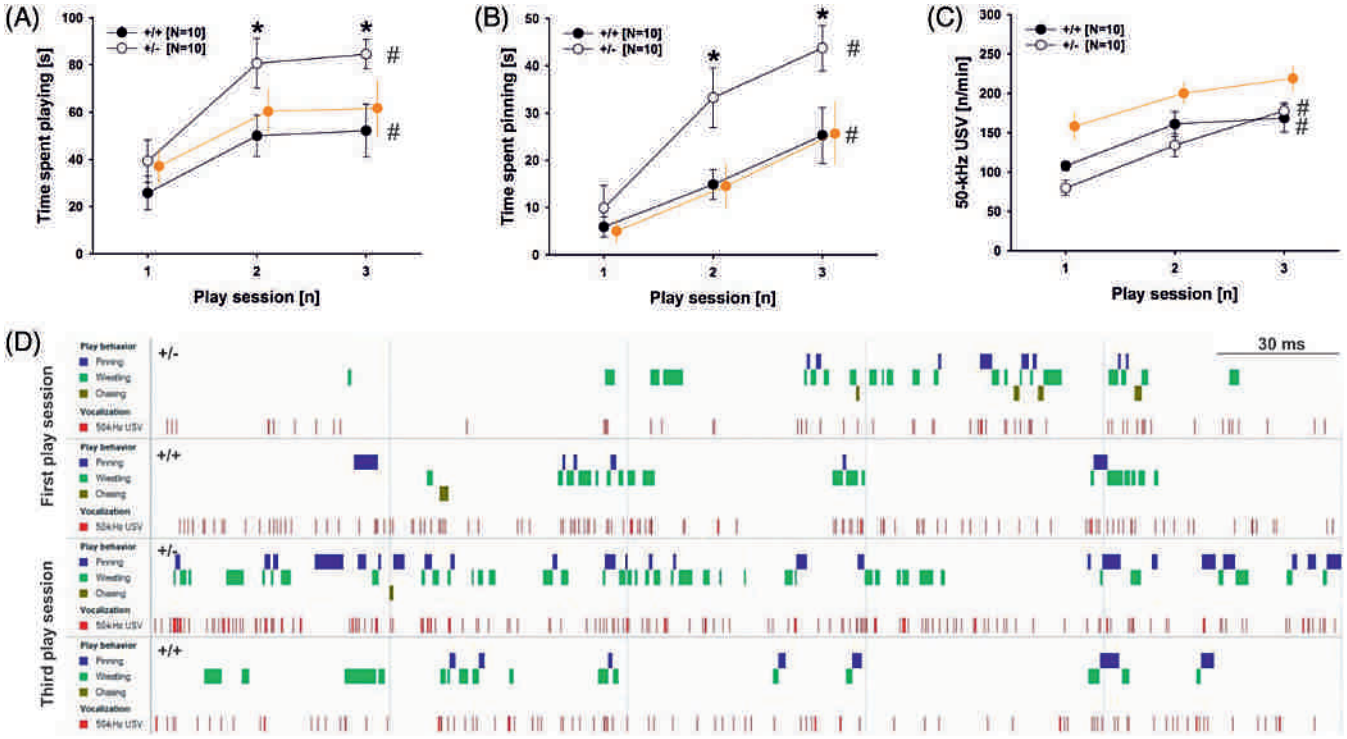

FIGURE 2 Rough-and-tumble play behavior and concomitant pro-social 50-kHz USV emission in *Cacna1c*$^{+/-}$ rats and *Cacna1c*$^{+/+}$ littermate controls. (A) Time spent playing; (B) time spent pinning; and (C) 50-kHz USV emission across the three play sessions in female *Cacna1c*$^{+/-}$ rats (white circles; $N = 10$) and *Cacna1c*$^{+/+}$ littermate controls (black circles; $N = 10$). For the sake of sex comparison, previously reported data in male *Cacna1c*$^{+/+}$ littermate controls are shown in orange.[45] (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 minutes play sessions. Data are presented as mean ± SEM. $^{\#}P < 0.050$ vs first play session; $*P < 0.050$ vs female *Cacna1c*$^{+/+}$ littermate controls

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 was increased, 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*$^{+/+}$ littermates 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 nonplay 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; Figure S1C,D). When comparing nonplay 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.[45] When comparing female and male *Cacna1c*$^{+/+}$ littermates, 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*$^{+/+}$ littermates, 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*$^{+/+}$ littermates starting from the second play session ($t_{18} = 2.430$; $P = 0.026$ and $t_{18} = 2.228$; $P = 0.039$; respectively).

3.3 | 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 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 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*$^{+/+}$ littermates both increased 50-kHz USV during playful interactions between test days ($t_9 = 14.219$; $P < 0.001$ and $t_9 = 3.638$; $P = 0.005$; respectively).

Contrary to females, *Cacna1c*$^{+/-}$ males emitted fewer 50-kHz USV than *Cacna1c*$^{+/+}$ littermates while engaged in playful interactions, as previously reported.[45] When comparing female and male *Cacna1c*$^{+/+}$ littermates, 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 interactions female *Cacna1c*$^{+/+}$ littermates vocalized less than male *Cacna1c*$^{+/+}$ littermates (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*$^{+/+}$ littermates. Specifically, while no difference in 50-kHz USV emitted during the anticipation phase was seen (S: $F_{1,17} = 3.436$; $P = 0.081$), *Cacna1c*$^{+/-}$ females vocalized less than male *Cacna1c*$^{+/+}$ littermates during playful interactions (S: $F_{1,18} = 13.114$; $P = 0.002$), and this effect was observed during all three consecutive 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).

3.4 | 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 activity in females in the 50-kHz USV radial maze playback paradigm (Figure 3B). Specifically, social exploratory activity induced by playback of 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 activity 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 activity was specifically seen in response to playback of 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*$^{+/+}$ littermates showed a lower number of 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 detectable 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). As compared to baseline, both genotypes continued to display reduced total arm entries after playback (T: $F_{1,19} = 112.034$; $P < 0.001$ and $F_{1,19} = 35.082$; $P < 0.001$; respectively).

The increase in social exploratory activity seen during playback of pro-social 50-kHz USV was primarily driven by approach behavior toward the sound source, that is, 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*$^{+/+}$ littermates showed 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), whereas the opposite was true for the time spent distal (T: $F_{1,19} = 13.065$; $P = 0.002$ and $F_{1,19} = 5.535$; $P = 0.030$; respectively). This led to a clear 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). However, while genotypes did not differ during 50-kHz USV playback, genotype differences were seen in the minutes following playback (T: $F_{1,38} = 0.965$; $P = 0.332$; TxG: $F_{1,38} = 7.625$; $P = 0.009$; P: $F_{1,38} = 20.035$; $P < 0.001$; PxG: $F_{1,38} = 0.413$; $P = 0.524$; TxP: $F_{1,38} = 5.406$; $P = 0.026$; TxPxG: $F_{1,38} = 0.016$; $P = 0.901$; Figure 3D, right). Specifically, *Cacna1c*$^{+/+}$ littermates continued displaying a preference for proximal over distal arms (P: $F_{1,19} = 6.773$; $P = 0.017$) and spent more time proximal after playback than before (T: $F_{1,19} = 8.057$; $P = 0.011$). In contrast, no such preference was seen in *Cacna1c*$^{+/-}$ females (P: $F_{1,19} = 1.555$; $P = 0.228$), which also did not spend more time proximal after playback than before (T: $F_{1,19} = 0.406$; $P = 0.531$). Irrespective of genotype, time spent distal did not differ from baseline (T: $F_{1,19} = 0.220$; $P = 0.644$ and $F_{1,19} = 3.311$; $P = 0.085$; respectively).

Contrary to females, social approach in response to playback of pro-social 50-kHz USV was strongly dependent on genotype in males, with *Cacna1c*$^{+/-}$ males displaying lower levels of social approach than *Cacna1c*$^{+/+}$ littermates, as previously reported.[45] When comparing female and male *Cacna1c*$^{+/+}$ littermates, social exploratory activity induced by playback of 50-kHz USV did not differ, that is, in the increase in the total number of arm entries ($t_{38} = 1.417$; $P = 0.165$), and there was no difference in social approach, that is, in the increase in the time spent proximal ($t_{38} = 1.031$; $P = 0.309$). Behavioral

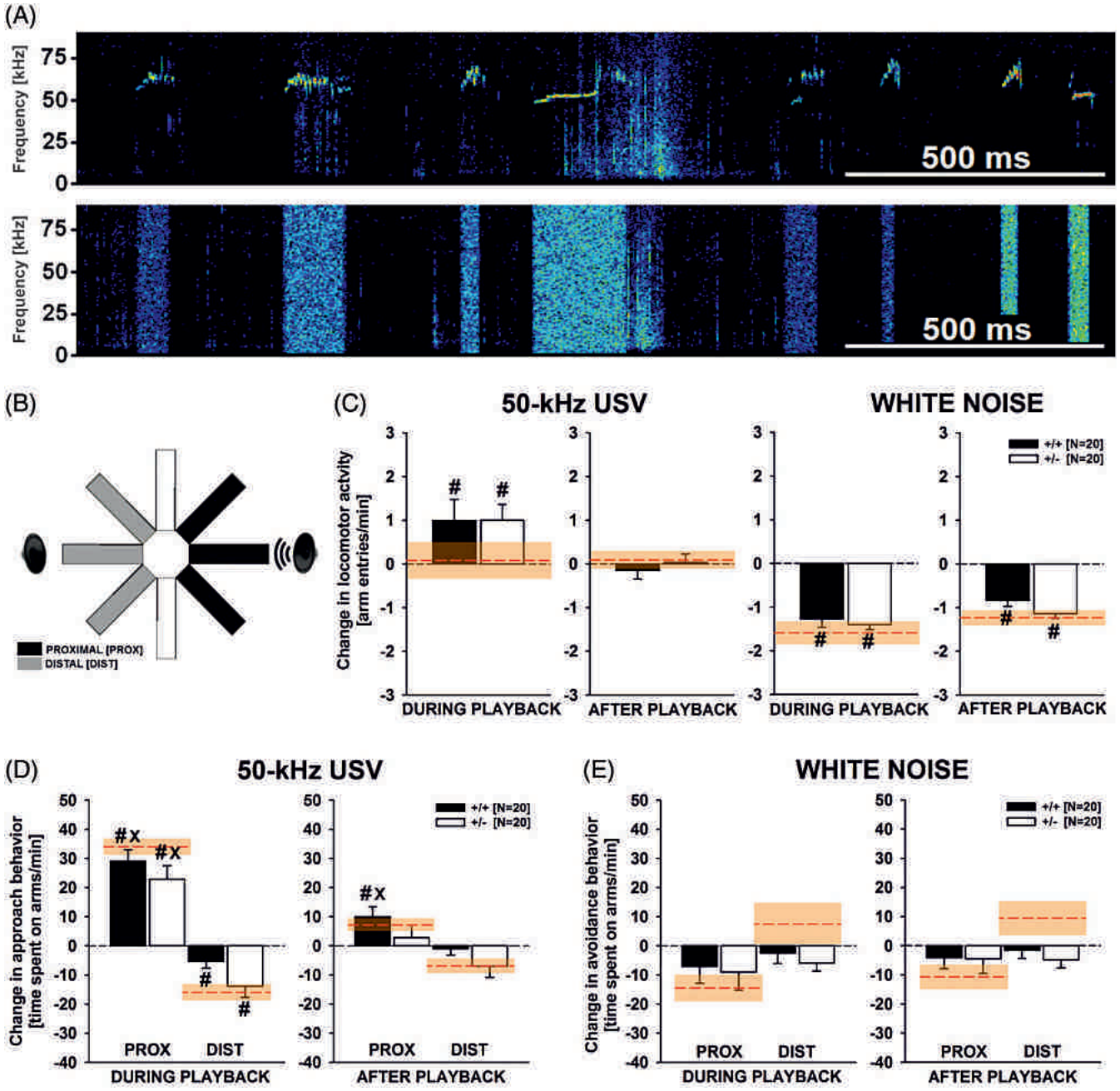

FIGURE 3 Social approach behavior evoked by pro-social 50-kHz USV playback in *Cacna1c*$^{+/-}$ rats and *Cacna1c*$^{+/+}$ littermate controls. (A) Exemplary spectrograms of acoustic stimuli used for playback, namely pro-social 50-kHz USV (upper panel) and time- and amplitude-matched White Noise (lower panel). (B) Schematic illustration of the radial maze used for playback depicting proximal (black), distal (gray), 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 black dashed line represents baseline levels. For the sake of sex comparison, previously reported data in male *Cacna1c*$^{+/+}$ littermate controls are shown in orange.[45] Data are presented as mean ± SEM. $^{\#}P < 0.050$ vs baseline levels; $^{x}P < 0.050$ vs distal

inhibition induced by playback of White Noise also did not differ ($t_{38} = 0.978$; $P = 0.334$). However, when comparing *Cacna1c*$^{+/-}$ females to male *Cacna1c*$^{+/+}$ littermates, exploratory behavior induced by playback of 50-kHz USV did not differ ($t_{38} = 1.664$; $P = 0.104$), yet social approach was lower in *Cacna1c*$^{+/-}$ females than in male *Cacna1c*$^{+/+}$ littermates ($t_{38} = 2.069$; $P = 0.045$). Behavioral inhibition induced by playback of White Noise did not differ ($t_{38} = 0.660$; $P = 0.513$).

3.5 | Response calls evoked by pro-social 50-kHz ultrasonic vocalizations

Some female recipient rats started to emit USV in response to playback of pro-social 50-kHz USV, while no USV were detected during White Noise exposure. Both *Cacna1c*$^{+/-}$ females and *Cacna1c*$^{+/+}$ littermate controls emitted more 50-kHz USV during 50-kHz USV playback than before (t_{19} = 2.668; P = 0.015 and t_{19} = 3.322; P = 0.004; respectively), although 50-kHz USV occurred only very rarely with less than one call per minute. In contrast, a substantial amount of 22-kHz USV was emitted in response to playback of 50-kHz USV (Figure 4A-C). This response was driven by *Cacna1c*$^{+/+}$ littermates 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 recipient rats emitted USV in response to 50-kHz USV but not White Noise playback. Both *Cacna1c*$^{+/-}$ males and *Cacna1c*$^{+/+}$ littermates 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*$^{+/+}$ littermates (t_{19} = 1.986; P = 0.062 and t_{19} = 3.329; P = 0.004; respectively; Figure 4D-F). 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*$^{+/+}$ littermates, USV in response to 50-kHz USV playback did not differ (all P-values >0.050). However, when comparing *Cacna1c*$^{+/-}$ females to male *Cacna1c*$^{+/+}$ littermates, 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).

3.6 | Repetitive and stereotyped patterns of behavior

Repetitive behavior in females was not affected by genotype, with similar levels of tail chasing (t_{38} = 0.591; P = 0.558; Figure S2A) and

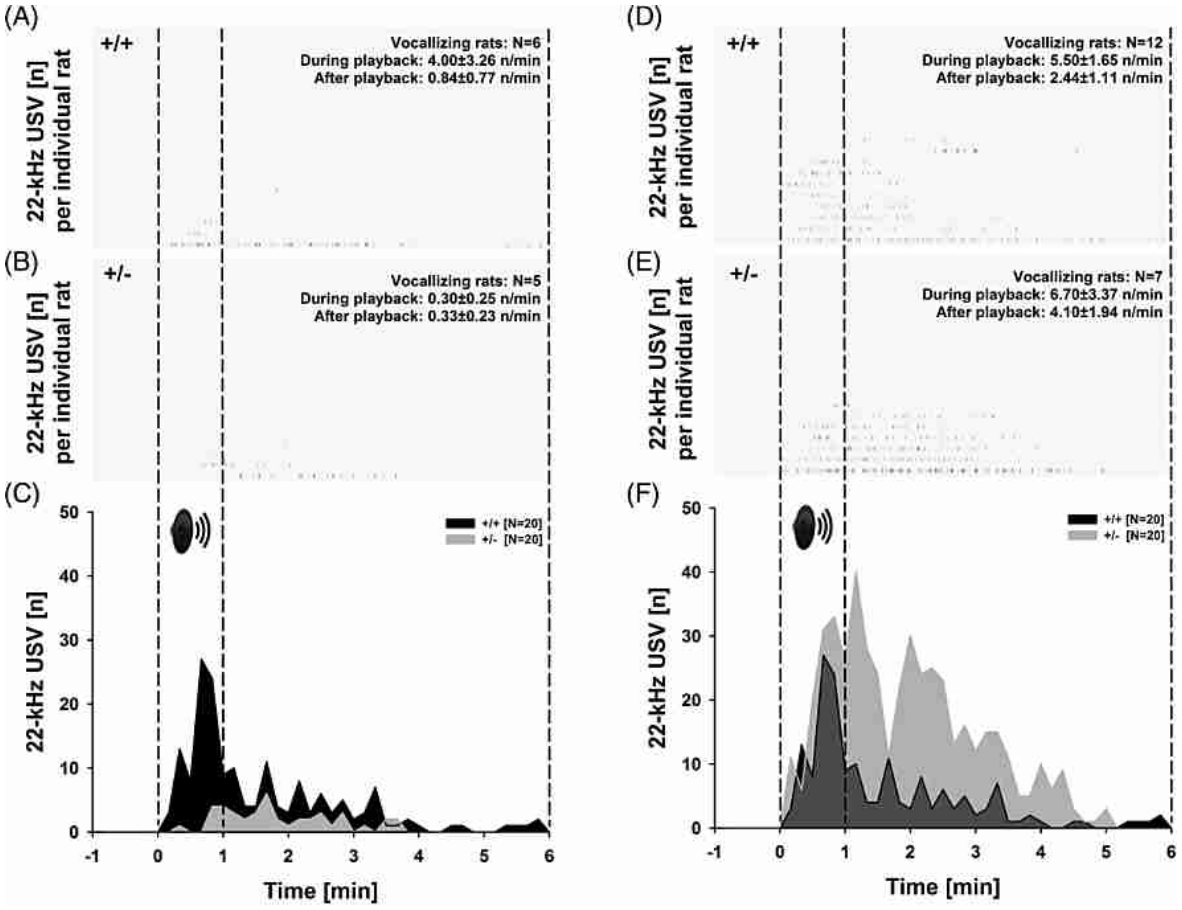

FIGURE 4 Response calls evoked by pro-social 50-kHz USV playback in *Cacna1c*$^{+/-}$ rats and *Cacna1c*$^{+/+}$ littermate controls. Emission of 22-kHz USV in (A) female *Cacna1c*$^{+/-}$ rats and (B) *Cacna1c*$^{+/+}$ littermate controls, with individual 22-kHz USV (red) per individual rat. (C) Total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

self-grooming behavior (t_{38} = 1.572; P = 0.124; Figure S2B), in line with findings obtained in males.[45] Locomotor activity was also not affected, and 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. Female and male *Cacna1c*$^{+/+}$ littermates did not differ (all P-values >0.050). Likewise, *Cacna1c*$^{+/-}$ females did not differ from male *Cacna1c*$^{+/+}$ littermates (all P-values >0.050), with the exception of self-grooming which was substantially higher in *Cacna1c*$^{+/-}$ females (t_{38} = 2.593, P = 0.006).

3.7 | Olfactory habituation and dishabituation

Finally, evidence for intact social and nonsocial 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 nonsocial odor stimuli, but clear dishabituation between familiar and novel social and nonsocial odor stimuli (Figure S3).

4 | DISCUSSION

In this study, we aimed at further exploring the role of *Cacna1c* in regulating behavioral phenotypes, focusing on sex-specific differences in social behavior and communication during the critical developmental period of adolescence in rats. In humans, the cross-disorder risk gene *CACNA1C* is implicated in the etiology of all major neuropsychiatric disorders, most notably ASD,[4–7] SCZ,[8–11] MDD,[8,12–14] and BPD.[15–18] Voltage-gated L-type Ca^{2+} channel functioning is also perturbed in Timothy syndrome (TS), a rare yet devastating disorder with symptoms partly similar to ASD.[86] TS typically arises from a sporadic SNP that generates a missense mutation (G406R) in *CACNA1C*.[6,7] Furthermore, SHANK scaffolding proteins, strongly associated with ASD,[87] have been implicated in the regulation of voltage-gated L-type Ca^{2+} channels, and thus mutations in the *SHANK* gene family could lead to $Ca_v1.2$ malfunctioning,[88] possibly contributing to ASD-related behavioral phenotypes. In fact, a G406R TS mouse model was reported to completely recapitulate an ASD-related behavioral phenotype and was 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,[89] yet social behavior findings remain equivocal.[90]

Despite evidence for sex-dependent effects of rs1006737 and other SNPs within *CACNA1C* on diagnosis, associated personality traits, and relevant resilience factors,[12,39,40] to our knowledge, sex-dependent effects of *Cacna1c* haploinsufficiency on relevant behavioral phenotypes in rodent models were rarely assessed, with a few exceptions.[12,81,82] Specifically, sex-dependent effects on social behavior and communication have not been studied in rats with genetic modifications targeting *Cacna1c* until now. Moreover, most currently available mouse studies focused on adult male mice, with no data being available for females.[89–94] This is particularly surprising because available evidence in humans points toward stronger effects in women than in men.[12,39,40] Moreover, the role of *Cacna1c* in regulating relevant behavioral phenotypes during the critical developmental period of adolescence, which is characterized by a particularly rich social behavior repertoire including social play behavior,[47–49] has not been extensively studied. In light of the evidence for sex-dependent effects in rodents[12,81,82] and humans,[12,39,40] and our previous report on reduced pro-social 50-kHz USV during rough-and-tumble play behavior in male *Cacna1c* haploinsufficient rats,[45] we expected prominent effects of *Cacna1c* haploinsufficiency in female rats.

Our present results show for the first time that partial depletion of *Cacna1c* leads to sex-dependent alterations in social behavior and pro-social 50-kHz ultrasonic communication 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 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 nonplayful social behaviors, with individual aspects, such as sniffing and physical contact, not differing between *Cacna1c*$^{+/-}$ females and *Cacna1c*$^{+/+}$ littermate controls.

Contrary to social play behavior, concomitant emission of pro-social 50-kHz USV did not differ between genotypes. *Cacna1c*$^{+/-}$ females and *Cacna1c*$^{+/+}$ littermate controls emitted comparable levels of 50-kHz USV while engaged in playful interactions. Because 50-kHz USV reflect positive affective states ("rat laughter")[61] associated with the rewarding nature of social play,[63] this would suggest that playful encounters were equally rewarding for *Cacna1c*$^{+/-}$ females as they were for *Cacna1c*$^{+/+}$ littermate controls. However, the substantial increase in pinning in *Cacna1c*$^{+/-}$ females speaks against equivalent reward levels between the two genotypes. Pinning and the close physical contact it offers is arguably one of the most rewarding components of playful interactions, as indicated by conditioned place preference[49] and surgical devocalization[79,95] experiments. Moreover, numerous studies support the notion that rats find tickling by an experienced human experimenter to be a highly rewarding experience, which stimulates them to emit very high numbers of 50-kHz USV.[65–68] Tickling is claimed to mimic the pinning sequence of playful 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.[66,68] Therefore, the more time spent pinning should naturally reinforce and increase the overall amount of reward to be gained from social play and thus 50-kHz USV.

Interestingly, however, even with the genotype difference in social play behavior, primarily driven by a more pronounced increase in pinning in *Cacna1c*$^{+/-}$ females across the three consecutive play sessions, there was no genotype difference in 50-kHz USV emission. 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. This suggests that the higher duration of playful interactions seen in *Cacna1c*$^{+/-}$ females on the second and third play sessions only grants them the same level of rewarding value gained by *Cacna1c*$^{+/+}$ littermate controls, suggesting reduced *liking*.[69] 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. The increase in 50-kHz USV emission by *Cacna1c*$^{+/-}$ females on the second and third play sessions, compared to the first day, is thus 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*$^{+/+}$ littermate controls. 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, most notably, during playful interactions engage in more pinning behavior.[96–98]

In males, there were no genotype differences in social play behavior, yet prominent differences in 50-kHz USV emission.[45] Across all three consecutive play sessions, *Cacna1c*$^{+/-}$ males consistently emitted less 50-kHz USV while engaged in playful interactions than *Cacna1c*$^{+/+}$ littermate controls. In an initial effort to link 50-kHz USV to specific components of the play repertoire, we conducted a series of temporal analyses applying high-resolution ethograms and further found that wrestling and chasing are typically associated with high numbers of 50-kHz USV, yet that this association is weak in *Cacna1c* haploinsufficient rats, with particularly low 50-kHz USV levels during wrestling. As with the females, similar levels of social play between genotypes thus led to reduced emission of 50-kHz USV in *Cacna1c*$^{+/-}$ males, again indicating that *Cacna1c*$^{+/-}$ rats do not derive as much reward from playful interactions as *Cacna1c*$^{+/+}$ littermate controls, possibly due to impaired *liking*.[69] However, *Cacna1c*$^{+/-}$ males did not compensate for low reward levels through increasing social play behavior above the levels seen in *Cacna1c*$^{+/+}$ littermate controls. As a consequence, 50-kHz USV emission remained consistently low in *Cacna1c*$^{+/-}$ males.

When comparing female and male *Cacna1c*$^{+/+}$ littermate controls employed in the same study, 50-kHz USV emission was clearly higher in males than females, although time spent playing was only slightly elevated in males as compared to females. This pattern is in line with a large number of other reports on sexually dimorphic rough-and-tumble play,[51] with male rats typically expressing a higher frequency of social play and engaging in rougher defense tactics, for example, more pinning behavior, than females.[52,53] Moreover, increased 50-kHz USV emission in males is consistent with a recent study.[74] In light of the more pronounced rough-and-tumble play in males, it appears particularly remarkable that *Cacna1c*$^{+/-}$ females did not only play more than female *Cacna1c*$^{+/+}$ but more even than male *Cacna1c*$^{+/+}$ littermate controls. However, not only the time engaged in rough-and-tumble play was higher in *Cacna1c*$^{+/-}$ females than in female and male *Cacna1c*$^{+/+}$ littermate controls. Social play in *Cacna1c*$^{+/-}$ females was also a lot rougher, as reflected in highly elevated levels of pinning behavior. Pinning behavior in *Cacna1c*$^{+/-}$ females was more than double as high as in male *Cacna1c*$^{+/+}$ littermate controls. Several studies have shown that females can be manipulated to display more masculinized play patterns, and similarly, males can be made to mirror more female-typical play patterns. For example, neonatal testosterone treatment creates a more masculinized playful repertoire in females,[53,99–101] with the amygdala appearing to be a key target region.[102] Conversely, castration and blocking testosterone receptors in males during early development creates female-typical social play.[99,103,104] However, in all such cases of masculinized rough-and-tumble play, females reached male play levels but did not exceed them. *Cacna1c* haploinsufficiency in females, in contrast, led to hypermasculinization, with rough-and-tumble play behavior, in general, and pinning behavior, in particular, being even higher than in males. We are not aware of other experimental manipulations that led to a similar hypermasculinization of rough-and-tumble play in juvenile female rats. We, therefore, believe that it is important to gain a better understanding of what is driving the hypermasculinization phenotype. Future studies on the effects of genotypes present in the home cage by comparing same-genotype and mixed-genotype housing might be particularly promising.

Besides emission of 50-kHz USV in the sender, partial *Cacna1c* depletion altered the behavioral responses elicited by playback of pro-social 50-kHz USV. Specifically, consistent with previous findings,[105] playback of 50-kHz USV led to social exploratory activity and approach behavior in females, with *Cacna1c*$^{+/-}$ females and *Cacna1c*$^{+/+}$ littermate controls displaying a clear preference for the sound source emitting 50-kHz USV. 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.[45] Although both *Cacna1c*$^{+/-}$ males and *Cacna1c*$^{+/+}$ littermate controls displayed social approach in response to playback of 50-kHz USV, the acute increase in time spent in proximity to the sound source was stronger in *Cacna1c*$^{+/+}$ littermate controls than in *Cacna1c*$^{+/-}$ males. Moreover, like in females, male *Cacna1c*$^{+/+}$ littermate controls kept searching for a conspecific and continued displaying a preference in the minutes following 50-kHz USV playback, whereas no clear preference was evident in *Cacna1c*$^{+/-}$ males. Social approach toward playback of pro-social 50-kHz USV reflects the motivation, that is, *wanting*, for social contact, typically expressed as the effort invested to get access to a social reward.[69] Together, our present findings thus further corroborate the idea that *Cacna1c* haploinsufficiency in rats might lead to less *wanting* in addition to the reduction in the *liking* component associated with rough-and-tumble play.[69]

Contrary to 50-kHz USV playback, White Noise led to strong behavioral inhibition in females irrespective of genotype, consistent with data obtained in males.[45] In males, however, the response evoked by White Noise also included avoidance behavior, with *Cacna1c*$^{+/+}$ littermate controls but not *Cacna1c*$^{+/-}$ males moving away from the sound source, a response pattern not evident in females. Yet the most striking sex difference was the lack of increased social

exploratory activity in response to playback of 50-kHz USV in males. Females, in contrast, displayed clearly elevated levels of social exploratory activity when exposed to playback of 50-kHz USV, in line with previous reports on particularly strong responses in females.[105]

In conjunction with the overt behavioral responses, that is, social exploratory activity and approach behavior, playback of pro-social 50-kHz USV stimulated the production of USV in recipient rats. While some recipient rats started to emit USV in response to 50-kHz USV playback, no USV were detected during White Noise exposure. Both *Cacna1c*$^{+/-}$ females and *Cacna1c*$^{+/+}$ littermate controls emitted more 50-kHz USV during 50-kHz USV playback than before, although 50-kHz USV occurred only very rarely. In contrast, a substantial amount of 22-kHz USV was emitted in response to playback of 50-kHz USV. This response was driven by *Cacna1c*$^{+/+}$ littermate controls, yet characterized by large variability. As in females, both *Cacna1c*$^{+/-}$ males and *Cacna1c*$^{+/+}$ littermate controls emitted more 50-kHz USV during 50-kHz USV playback than before, in line with previous studies in males.[60,106] While 50-kHz USV occurred only very rarely, a substantial amount of 22-kHz USV was detected in both *Cacna1c*$^{+/-}$ males and *Cacna1c*$^{+/+}$ littermate controls, again in line with previous studies in males.[60,106] Moreover, and contrary to females, 22-kHz USV emission remained high following 50-kHz USV playback in both genotypes. Together, this shows that 22-kHz USV were particularly low in *Cacna1c*$^{+/-}$ females. In previous studies, 22-kHz USV were linked to frustration. For instance, it was shown that 22-kHz USV occur if an expected sucrose reward is not delivered[107] and it was suggested that 22-kHz USV in response to 50-kHz USV playback occur because the recipient rats cannot reach the sound source.[106] In fact, the rats that tried most vigorously to reach the sound source emitted particularly high numbers of 22-kHz USV.

The effects of *Cacna1c* haploinsufficiency on rough-and-tumble play and pro-social 50-kHz ultrasonic communication obtained in rats are partly in line with what was found in *Cacna1c* mouse studies. For instance, it was reported that adult male forebrain *Cacna1c*$^{-/-}$ mice do not prefer a conspecific to an object in the widely applied three-chambered social approach assay.[91,93] Consistently, *Cacna1c* knockdown specifically in the prefrontal cortex[93] but not the nucleus accumbens[94] likewise led to lack of sociability. In constitutive *Cacna1c*$^{+/-}$ mice, the *pendant* to the rat model applied in this study, however, no evidence for deficits in social functioning was obtained,[91] with one study even reporting enhanced sociability in a newly developed social home cage assay.[89] Finally, further evidence for *Cacna1c* being involved in the regulation of socio-affective information processing stems from an observational fear learning study.[92] Vicarious freezing was found to be markedly reduced upon local $Ca_v1.2$ depletion in the anterior cingulate cortex. Of note, however, all of the aforementioned studies investigating social behavior in mice with genetic modifications targeting *Cacna1c* have focused solely on males, thus, it is not known whether the effects of *Cacna1c* haploinsufficiency are sex-dependent. Because it was reported that constitutive *Cacna1c*$^{-/-}$ mice are not viable,[108] we made no attempt to generate *Cacna1c*$^{-/-}$ rats.

What is driving the sex-dependent effects of *Cacna1c* haploinsufficiency on juvenile social play behavior and pro-social 50-kHz ultrasonic communication in rats? Notably, $Ca_v1.2$ protein levels were reduced by slightly more than 50% in the brain of *Cacna1c*$^{+/-}$ females. As a similar reduction was also seen *Cacna1c*$^{+/-}$ males,[45] $Ca_v1.2$ protein levels do not explain the sex-dependent effects of *Cacna1c* haploinsufficiency. Interestingly, however, it was reported that $Ca_v1.2$ is a target of estradiol in vivo and that estradiol directly potentiates Ca^{2+} influx via $Ca_v1.2$ through the dihydropyridine binding site.[109] Moreover, estradiol treatment was found to prevent an age-related increase in $Ca_v1.2$ current in hippocampal neurons and led to a reduction in $Ca_v1.2$ mRNA.[110]

Another potential candidate appears to be the neurotransmitter dopamine (DA). DA has been strongly associated with reward processing[111,112] and 50-kHz ultrasonic communication.[113,114] Importantly, DA signaling is profoundly altered in mice with genetic modifications targeting *Cacna1c*,[115] with evidence for sex-dependency of *Cacna1c* depletion effects.[12] 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 ventral tegmental area and the medial forebrain bundle,[116–118] with 50-kHz USV emission and phasic DA release in the nucleus accumbens being time-locked.[118] Moreover, 50-kHz USV emission can be induced pharmacologically by systemic administration of psychostimulants resulting in enhanced DA levels in the synaptic cleft, most notably amphetamine.[70,71,76,119] Local administration of amphetamine in the nucleus accumbens results in a massive increase in 50-kHz USV emission.[120–122] Besides 50-kHz USV emission in the sender, social approach elicited by playback of 50-kHz USV evokes phasic DA release in the nucleus accumbens of the recipient rat[123] and enhancing DA signaling by amphetamine treatment results in more pronounced 50-kHz USV responsivity.[76] In mice with genetic modifications targeting *Cacna1c*, ample evidence indicates that DA signaling is impaired. For instance, it was found that hyperlocomotion induced by amphetamine treatment is reduced in adult male and female *Cacna1c*$^{+/-}$ mice as compared to *Cacna1c*$^{+/+}$ littermate controls, with greater attenuation of amphetamine-induced hyperlocomotion in females than in males.[12] Moreover, reduced hyperlocomotion in response to methamphetamine in *Cacna1c*$^{+/-}$ mice was found on a wide range of genetic backgrounds.[124] Extending these findings, 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.[125] By means of fast-scan cyclic voltammetry, it was further shown 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 demonstrating that $Ca_v1.2$ regulate DAergic burst firing in the ventral tegmental area.[126] 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 emission and/or responsivity in *Cacna1c* haploinsufficient rats. This is of considerable translational interest because, for instance, 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.[127]

Importantly, in this study, genotype effects on social behavior and ultrasonic communication were not associated with deficits in behavioral activity and motor functions. Moreover, confounding olfactory deficits can be ruled out, as evidenced by the olfactory habituation and dishabituation paradigm, with both genotypes showing the expected zig-zag-shaped pattern, reflecting intact olfactory abilities. This is consistent with a previous study in *Cacna1c*$^{+/-}$ mice where the time to find food in the hidden cookie test was not affected by *Cacna1c* haploinsufficiency.[12] *Cacna1c*$^{+/-}$ rats further showed no repetitive behaviors, such as tail chasing or self-grooming, indicating that partial *Cacna1c* depletion does not result in the characteristic repetitive behaviors seen in human ASD and relevant rodent models.[128] 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[129] and marble-burying.[89] Together, 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 future studies, it will be important to perform additional assays with relevance to other neuropsychiatric disorders, such as SCZ, MDD, and BPD, and to study sex-specific effects on potential neurobiological mechanisms, including alterations in gene regulation.[130]

In humans, genome-wide association studies and other genetic approaches have identified a cluster of noncoding SNPs within *CACNA1C* to be strongly associated with all major neuropsychiatric disorders, including ASD,[4–7] SCZ,[8–11] MDD,[8,12–14] BPD.[15–18] However, the mechanisms through which these SNPs confer susceptibility are not entirely clear. It was suggested that SNPs within 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.[131] 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[131] and BPD[132] patients. Moreover, *CACNA1C* hypermethylation was recently reported in BPD patients carrying the rs1006737 risk variant, suggesting that the regulatory effect of the noncoding risk variants involve a shift in DNA methylation, ultimately resulting in reduced protein expression.[133] Changes in the epigenetic regulation of *CACNA1C* were also linked to ASD.[134]

Consistent with our genetic *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.[135] Notably, however, genotype effects on personality traits were reported to be strongly sex-dependent and simple non-sex-specific genotype-phenotype analyses did not reveal any association in another study.[40] Yet strong sex-specific associations with rs1006737 were detected for neuroticism, sense of coherence, and dispositional optimism, with prominent genotype x sex interaction effects and opposite associations with rs1006737 in women and men. Similar findings were obtained for perceived social support as well as for depressive symptoms. Of note, very recently strong sex-dependent effects were also obtained for another *CACNA1C* risk variant, with greater hostility and reduced neuronal activity in frontolimbic regions during cognitive processing in women.[136] Secondly, the *CACNA1C* risk variant rs1006737 impairs socio-affective information processing in humans, slowing down facial emotion recognition in healthy individuals[137] and reducing accuracy in BPD patients.[28] 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,[34–36] possibly due to alterations in brain connectivity.[138,139] Finally, verbal fluency is reduced in rs1006737 risk allele carriers, hindering language production on a semantic level.[38] Together, low extraversion and hostility with slowed and incorrect facial emotion recognition and impaired language production might thus impair social interaction and competence in human risk allele carriers. Interestingly, as suggested by the present and previous rodent findings,[12,124–126] rs1006737 is also linked to alterations in reward processing in humans. For instance, increased amygdala reactivity in response to monetary reward in rs1006737 risk allele carriers was observed.[33] To our knowledge, however, social reward has not yet been studied in rs1006737 risk allele carriers.

5 | CONCLUSION

In summary, our results show for the first time that partial depletion of *Cacna1c* leads to sex-dependent alterations in social behavior and communication in rats. In females, *Cacna1c* haploinsufficiency led to hypermasculinization, with rough-and-tumble play behavior, in general, and pinning behavior, in particular, being even higher than in males without affecting concomitant 50-kHz USV. In males, in contrast, rough-and-tumble play behavior was not altered, yet emission of 50-kHz USV was diminished following partial *Cacna1c* depletion. This may suggest that *Cacna1c* haploinsufficient rats derive less reward from playful interactions. The behavioral responses elicited by playback of 50-kHz USV were reduced upon partial *Cacna1c* depletion in both sexes, indicating that *Cacna1c* haploinsufficiency has detrimental effects on pro-social 50-kHz ultrasonic communication in both sender and recipient rat. Together, partial *Cacna1c* depletion effects on rough-and-tumble play behavior were prominent in females but absent in males, whereas pro-social 50-kHz ultrasonic communication was particularly affected in males with weaker effects in females. It thus can be concluded that *Cacna1c* plays a prominent sex-dependent role in regulating juvenile rat social play behavior and pro-social 50-kHz ultrasonic communication with relevance to sex-specific effects seen in neuropsychiatric disorders.

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CONFLICTS OF INTEREST

The authors declare no competing interests.

Author contributions

M.W. conceived the study; T.K., M.B., and S.M. performed the experiments; T.K. and M.W. analyzed the data; T.K. and M.W. wrote the manuscript with the help of all other authors; S.W., M.R., C.C., R.S., and M.W. acquired funding.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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

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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 seven 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.

1. 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; Dao et al., 2010; Green et al., 2010), and schizophrenia (SCZ; Nyegaard et al., 2010, Ripke et al., 2014; for review see Kabir, Lee, & Rajadhyaksha, 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 (Ca^{2+}) channel Cav1.2, which regulates depolarization-dependent Ca^{2+} 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, Lee, &

Abbreviations: BPD, bipolar disorder; MDD, major depressive disorder; SCZ, schizophrenia; SNP, single-nucleotide polymorphism; GWAS, genome-wide association study; *Cacna1c*$^{+/-}$, heterozygous *Cacna1c*; *Cacna1c*$^{+/+}$, wildtype *Cacna1c*; PND, postnatal day

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Rajadhyaksha, 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 (Goldberg & Chengappa, 2009; Kurtz & Gerraty, 2009), low processing speed and negative affective bias in MDD (Hammar, Lund, & Hugdahl, 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, Giakoumaki, Georgakopoulos, Robakis, and Bitsios (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, Sze, White, & Murphy, 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, Wöhr, & Alenina, 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.

2. Materials and methods

2.1. 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 460,649–460,652 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., 2018). 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 × 38 × 20 cm, length × width × 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).

2.2. 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).

2.3. 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 127-140 ± 21. 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., 2018). 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.

2.4. 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 × 60 × 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 × 5 × 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 lx) conditions.

2.5. Spatial and reversal learning

Around PND 130-140, 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 × 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 × 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 × 150 × 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 lx) conditions.

2.6. 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 (1–7) 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).

3. Results

3.1. 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 × 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) [Fig. 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 × 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 ($t_9 = 1.386$; $p = 0.199$) [Fig. 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 × genotype: $F_{6,108} = 0.791$; $p = 0.579$), with both genotypes making fewer wrong entries on day 7 compared to day 1 (*Cacna1c*$^{+/+}$: $t_9 = 3.515$; $p = 0.007$; *Cacna1c*$^{+/-}$: $t_9 = 4.996$; $p = 0.001$) [Fig. 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 × 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 ($t_9 = 1.684$; $p = 0.126$) [Fig. 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 × genotype: $F_{6,108} = 0.398$; $p = 0.879$) even when comparing the first and last day of testing (*Cacna1c*$^{+/+}$: $t_9 = -0.330$; $p = 0.749$; *Cacna1c*$^{+/-}$: $t_9 = -0.079$; $p = 0.939$) [Fig. 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 × 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) [Fig. 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: $t_{17} = -1.627$; $p = 0.061$) [Fig. 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 × 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) [Fig. 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 × 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 (*Cacna1c*$^{+/+}$: $t_9 = 6.070$; $p < 0.001$; *Cacna1c*$^{+/-}$: $t_9 = 0.963$; $p = 0.361$) [Fig. 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 × 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) [Fig. 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 × genotype: $F_{6,108} = 1.733$; $p = 0.120$) where both genotypes improved from day 1 to the last spatial learning day (*Cacna1c*$^{+/+}$: $t_9 = 5.040$; $p = 0.001$; *Cacna1c*$^{+/-}$: $t_9 = 3.143$; $p = 0.012$) [Fig. 2D, left]. Regarding WM errors, no progress was seen for either genotype between day 1 and day 7 (*Cacna1c*$^{+/+}$: $t_9 = 0.699$; $p = 0.502$; *Cacna1c*$^{+/-}$: $t_9 = -0.712$; $p = 0.495$), 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 × genotype: $F_{6,108} = 0.462$; $p = 0.835$) [Fig. 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 × genotype: $F_{6,108} = 0.268$; $p = 0.951$) and on day 7 compared to day 1 (*Cacna1c*$^{+/+}$: $t_9 = 3.459$; $p = 0.007$; *Cacna1c*$^{+/-}$: $t_9 = 3.428$; $p = 0.008$). Here, *Cacna1c*$^{+/-}$ females performed better than their *Cacna1c*$^{+/+}$ counterparts ($F_{1,18} = 8.836$; $p = 0.008$) [Fig. 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: $t_{18} = -0.676$; $p = 0.254$) [Fig. 4B].

3.2. 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 × 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 (*Cacna1c*$^{+/+}$: $t_9 = 3.884$; $p = 0.004$; *Cacna1c*$^{+/-}$: $t_9 = 4.140$; $p = 0.003$) [Fig. 1A, right]. The number of average entries

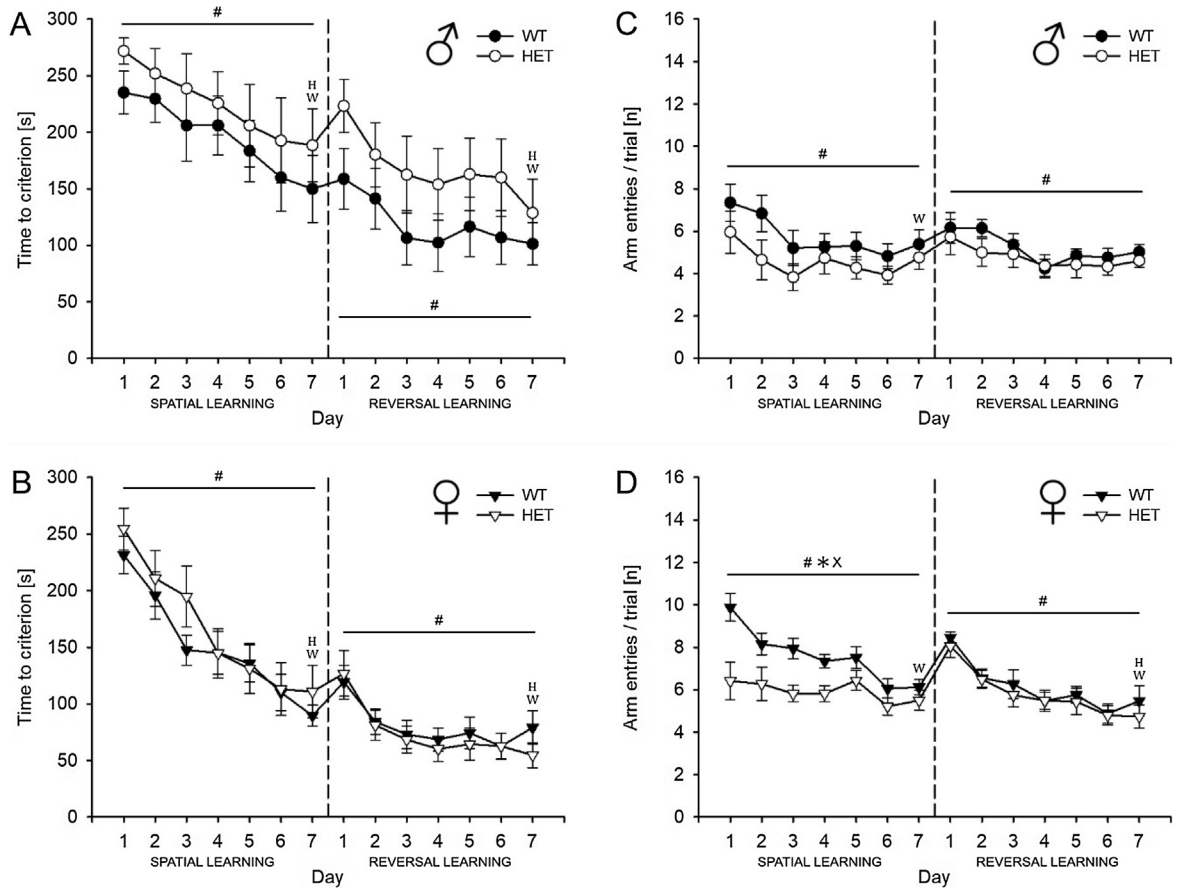

Fig. 1. All experimental groups required less time and fewer entries across training days, in females with initial hypo-activity in heterozygous *Cacna1c*$^{+/-}$ and a steeper decline in wildtype *Cacna1c*$^{+/+}$ during spatial learning. (a) (b) Average latency during spatial (left part) and reversal 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 300 s. 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 × genotype). W = $p < 0.05$ (comparison day 1 and 7 in *Cacna1c*$^{+/+}$). H = $p < 0.05$ (comparison day 1 and 7 in *Cacna1c*$^{+/-}$).

did not reduce in the same way between the first and last day (*Cacna1c*$^{+/+}$: $t_9 = 1.347$; $p = 0.211$; *Cacna1c*$^{+/-}$: $t_9 = 1.138$; $p = 0.284$), 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 × genotype: $F_{6,108} = 0.450$; $p = 0.843$) [Fig. 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 × 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 (*Cacna1c*$^{+/+}$: $t_9 = 3.760$; $p = 0.004$; *Cacna1c*$^{+/-}$: $t_9 = 4.214$; $p = 0.002$) [Fig. 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 × genotype: $F_{6,108} = 0.252$; $p = 0.958$) where both genotypes made fewer errors by day 7 (*Cacna1c*$^{+/+}$: $t_9 = 5.071$; $p = 0.001$; *Cacna1c*$^{+/-}$: $t_9 = 3.310$; $p = 0.009$) [Fig. 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 × genotype: $F_{6,108} = 0.445$; $p = 0.847$). Neither group made fewer WM errors after reversal learning than at the very start (*Cacna1c*$^{+/+}$: $t_9 = -1.100$; $p = 0.300$; *Cacna1c*$^{+/-}$: $t_9 = -0.693$; $p = 0.506$) [Fig. 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 × 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) [Fig. 3C, right].

In the probe trial following reversal learning, both genotypes could distinguish between the previously rewarded arms and the non-rewarded arms (*Cacna1c*$^{+/+}$: $t_8 = 1.973$; $p = 0.042$; *Cacna1c*$^{+/-}$: $t_9 = 3.701$; $p = 0.003$), 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 ($t_{17} = -2.231$; $p = 0.020$) [Fig. 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 (*Cacna1c*$^{+/+}$: $t_9 = 2.874$; $p = 0.018$; *Cacna1c*$^{+/-}$: $t_9 = 3.968$; $p = 0.003$), 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 × genotype: $F_{6,108} = 0.915$; $p = 0.487$) [Fig. 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 × 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 (*Cacna1c*$^{+/+}$: $t_9 = 3.411$; $p = 0.008$; *Cacna1c*$^{+/-}$: $t_9 = 5.219$; $p = 0.001$) [Fig. 1D, right].

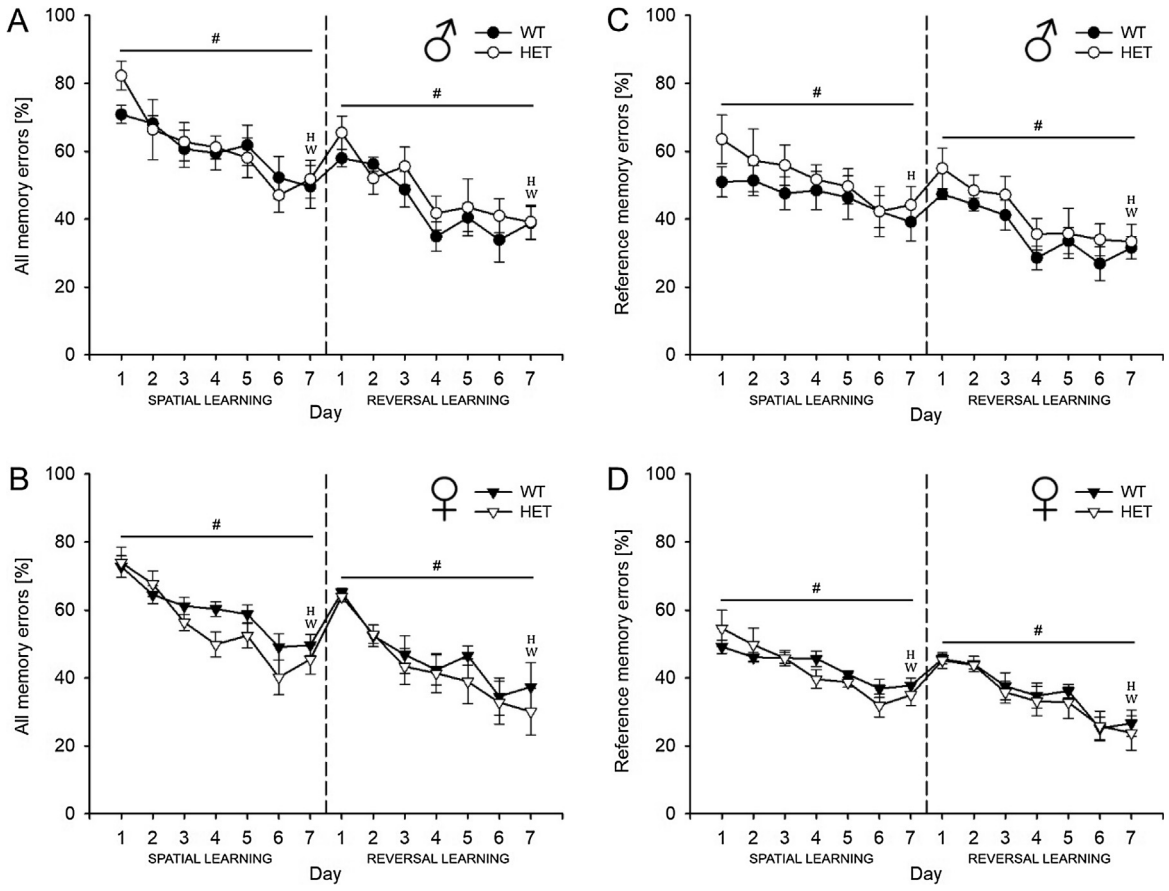

Fig. 2. Intact spatial and reversal learning abilities in all experimental groups. (a) (b) Percentage of all wrong arm entries relative to total number of entries in wildtype *Cacna1c*$^{+/+}$ and *Cacna1c*$^{+/-}$ depicted for males (a) and females (b). All experimental groups reduced their total error 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*$^{+/-}$).

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 × genotype: $F_{6,108} = 0.427$; $p = 0.860$), from day 1 to day 7 (*Cacna1c*$^{+/+}$: $t_9 = 3.626$; $p = 0.006$; *Cacna1c*$^{+/-}$: $t_9 = 4.787$; $p = 0.001$) [Fig. 2B, right], which was also true for RM errors (*Cacna1c*$^{+/+}$: $t_9 = 4.087$; $p = 0.003$; *Cacna1c*$^{+/-}$: $t_9 = 3.704$; $p = 0.005$) 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 × genotype: $F_{6,108} = 0.145$; $p = 0.990$) [Fig. 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 × 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) [Fig. 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 × 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 ($t_9 = 1.774$; $p = 0.110$) [Fig. 3D, right].

However, in the consecutive probe trial both genotypes displayed the ability to distinguish previously baited from non-baited arms (*Cacna1c*$^{+/+}$: $t_8 = 3.000$; $p = 0.009$; *Cacna1c*$^{+/-}$: $t_9 = 6.029$; $p < 0.001$), with a slightly more pronounced avoidance of the incorrect arms in *Cacna1c*$^{+/-}$ rats (baited: $t_{17} = 1.735$; $p = 0.051$ and non-baited: $t_{17} = -2.076$; $p = 0.027$) [Fig. 4D].

3.3. 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 [Fig. 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 (*Cacna1c*$^{+/+}$: $t_9 = -6.527$; $p < 0.001$; *Cacna1c*$^{+/-}$: $t_9 = -4.625$; $p < 0.001$). 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 ($t_9 = 2.963$; $p = 0.008$) [Fig. 5B].

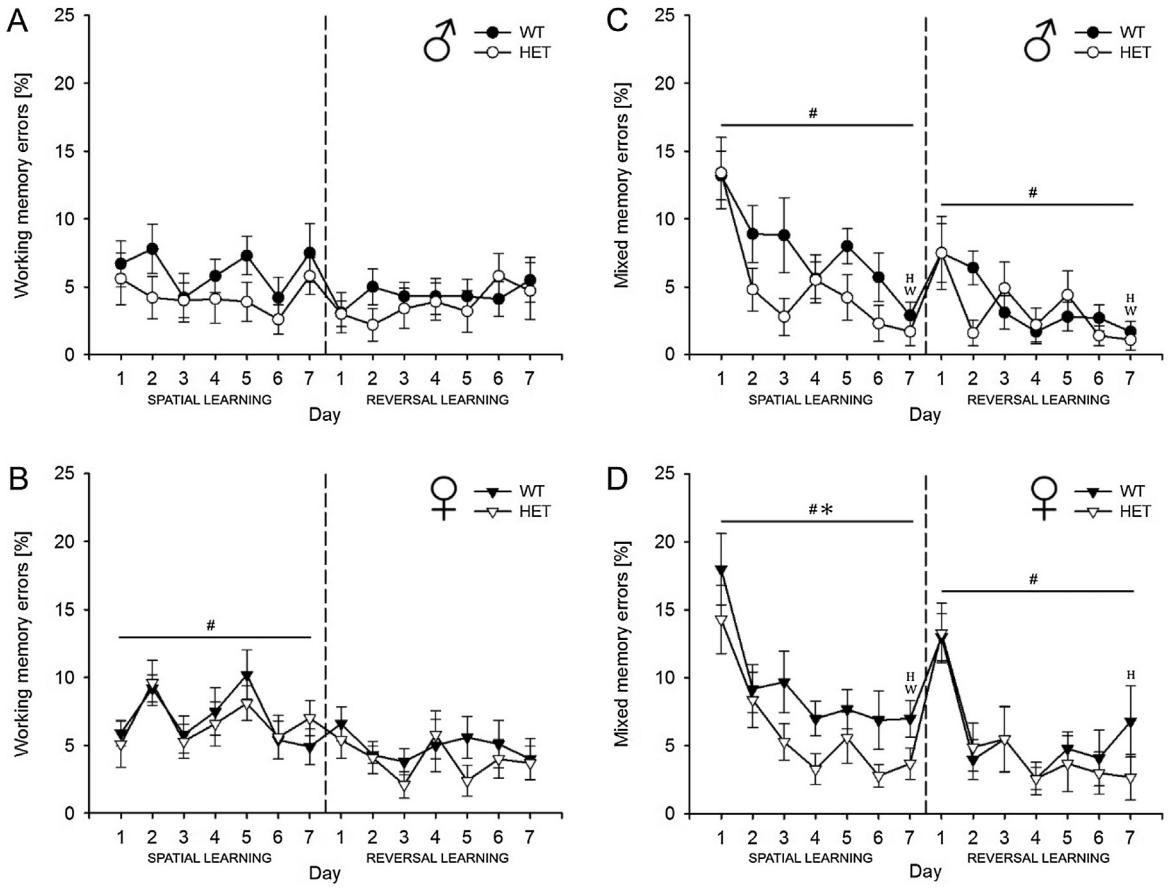

Fig. 3. No improvement in working memory performance, but declining mixed errors with better performance in heterozygous *Cacna1c*$^{+/-}$ females. (a) (b) Percentage of repeated entries into *rewarded* arms relative to total number of entries in wildtype *Cacna1c*$^{+/+}$ and *Cacna1c*$^{+/-}$ depicted 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*$^{+/-}$).

3.4. 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 (*Cacna1c*$^{+/+}$: $t_9 = 4.583$; $p < 0.001$; *Cacna1c*$^{+/-}$: $t_9 = 2.414$; $p = 0.020$) [Fig. 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 [Fig. 6C].

Females: The same holds true for females, where *Cacna1c*$^{+/+}$ and *Cacna1c*$^{+/-}$ rats preferred the higher baited arms during the spatial learning probe ($t_9 = 3.059$; $p = 0.007$ and $t_9 = 6.228$; $p < 0.001$; respectively) [Fig. 6B]. A preference for the higher baited arms was also seen during the reversal learning probe, irrespective of genotype (*Cacna1c*$^{+/+}$: $t_8 = 2.121$; $p = 0.034$; *Cacna1c*$^{+/-}$: $t_9 = 4.605$; $p < 0.001$) [Fig. 6D].

3.5. 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) [Fig. 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: $t_{38} = 0.104$; $p = 0.918$) [Fig. 7B, left] or trial 2 (males: $t_{38} = 0.731$; $p = 0.470$; females: $t_{37} = 1.582$; $p = 0.122$) [Fig. 7B, right].

4. 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 seven 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.

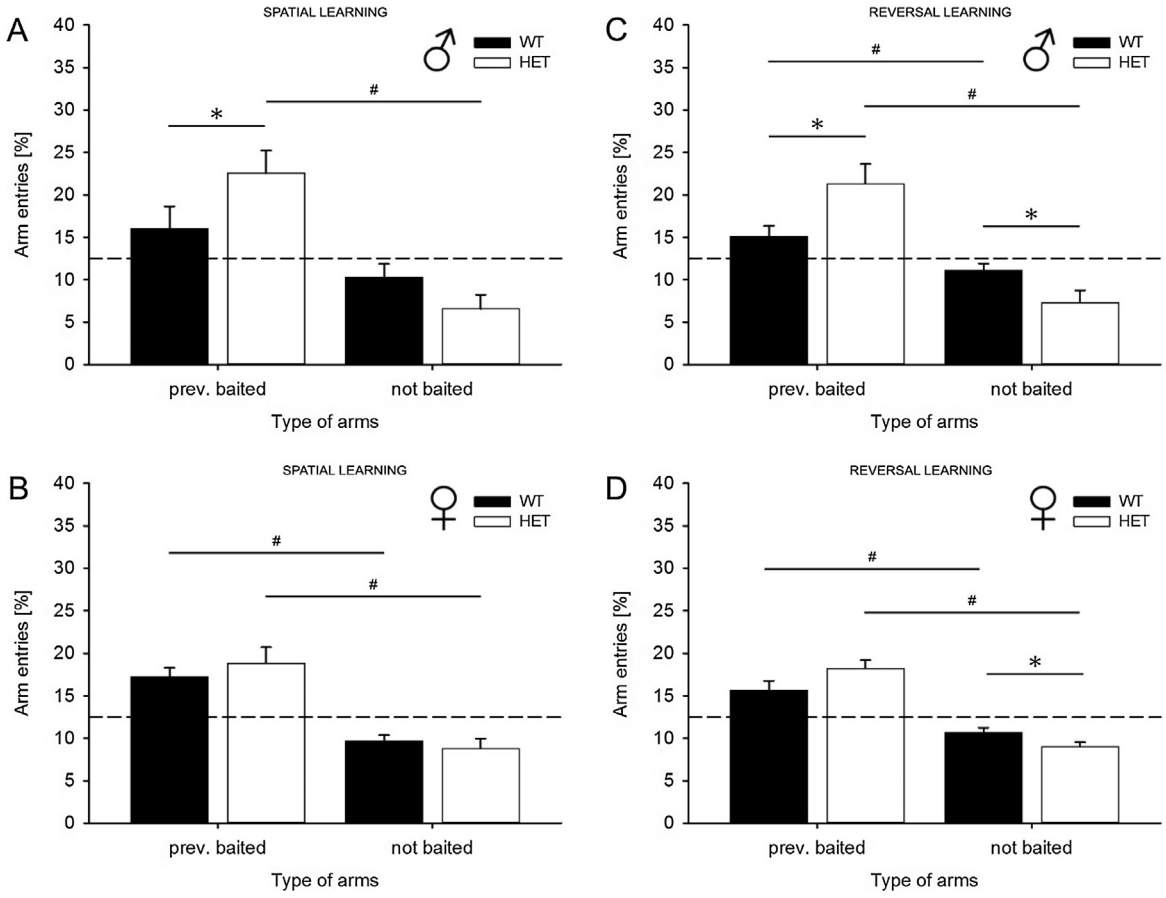

Fig. 4. Intact memory retention during probe trials with stronger memory traces in heterozygous *Cacna1c*$^{+/-}$ males. (a) (b) Percentage of arm entries into previously baited arms during spatial learning relative to all probe entries and adjusted for number of arms of the specific type in wildtype *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).

4.1. 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

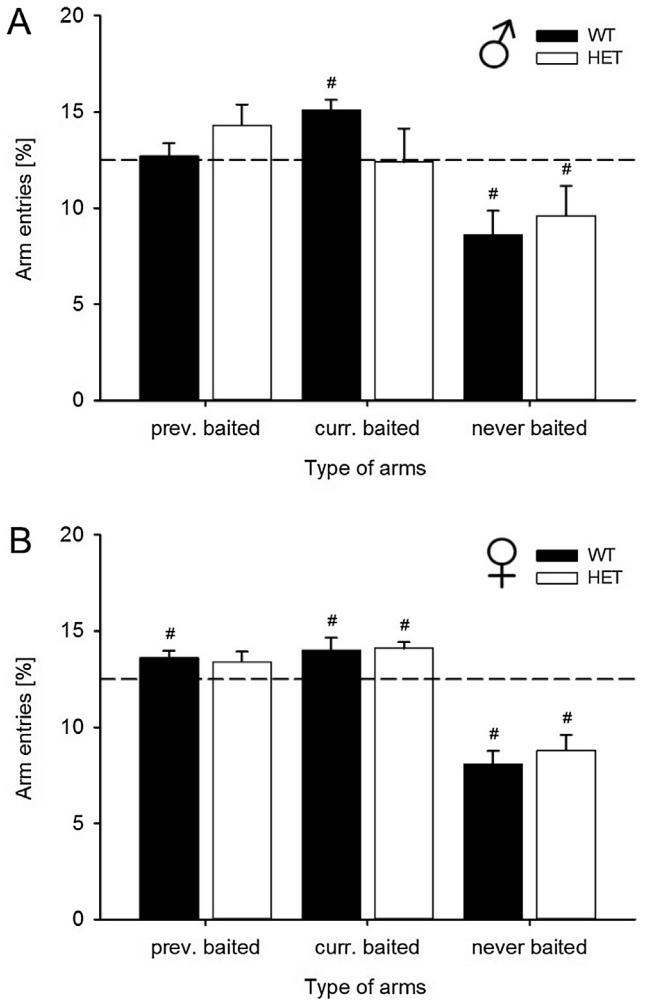

Fig. 5. Intact cognitive flexibility on reversal learning day 1 in wildtype *Cacna1c*$^{+/+}$ males and heterozygous *Cacna1c*$^{+/-}$ females, with perseveration tendencies in *Cacna1c*$^{+/-}$ males and *Cacna1c*$^{+/+}$ females. (a) (b) Percentage of entries made into previously rewarded arms from 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).

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, Bell, Fisher, and Murphy (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. (2018) 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. Kabitzke et al. (2017) had male wildtype and heterozygous *Cacna1c* 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 Kabitzke 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, Fischer, Rizzo, and Rajadhyaksha (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 (Temme et al., 2016; White et al., 2008).

4.2. 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,

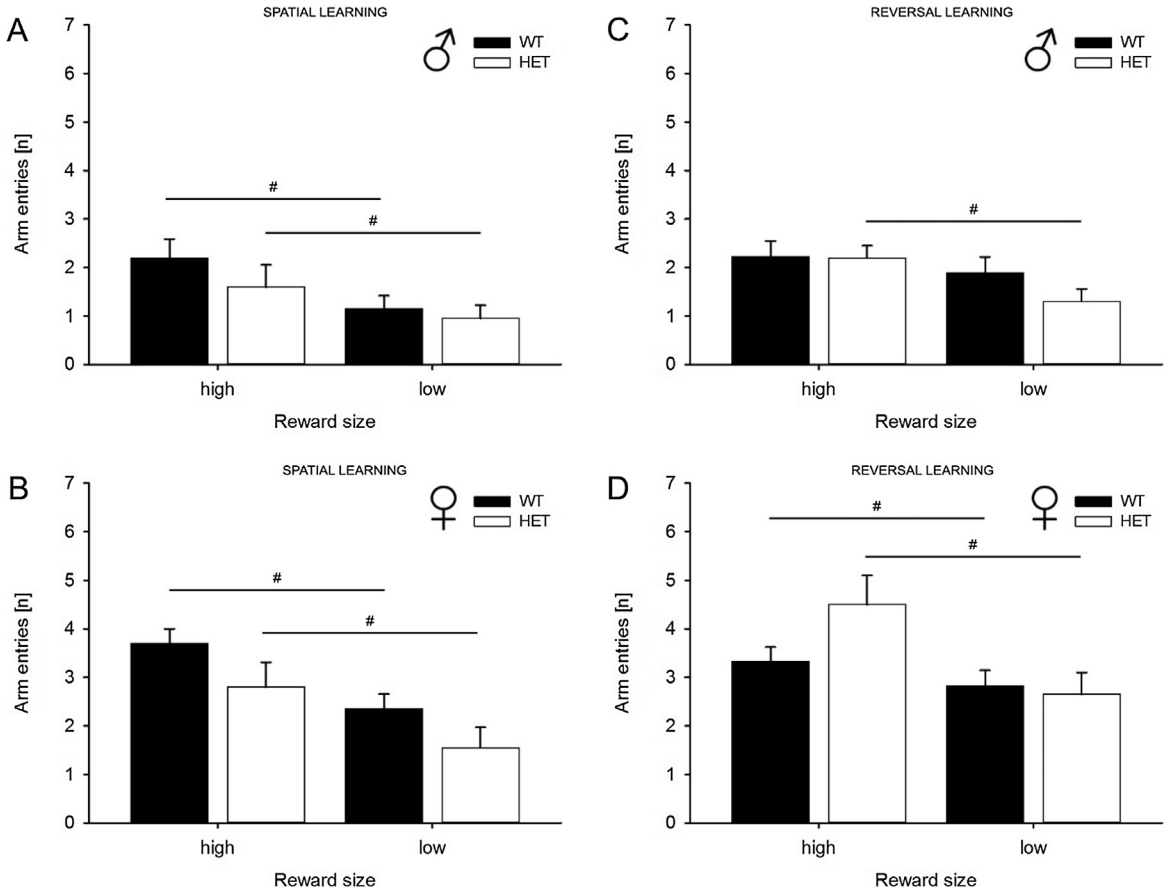

Fig. 6. Intact reward sensitivity in wildtype *Cacna1c*$^{+/+}$ and heterozygous *Cacna1c*$^{+/-}$ as assessed during probe trials. Number of entries made into the arm previously containing six pellets (high) and arms with one pellet during learning (low, average of 2 arms) during spatial learning probe 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).

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

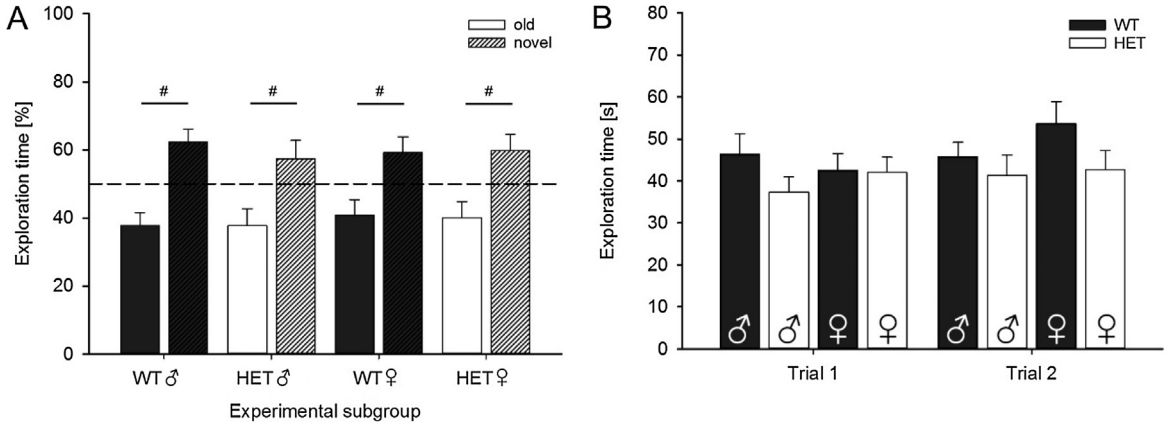

Fig. 7. Intact novel object recognition in wildtype *Cacna1c*$^{+/+}$ *and heterozygous Cacna1c*$^{+/-}$. (a) Depicted is the time spent sniffing (percentage of total exploration in trial 2) the novel object (patterned bars) vs. the familiar object (blank bars). All experimental groups could distinguish 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). * = $p < 0.05$.

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. (2018) 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, Kabitzke 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.

4.3. 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, Heerey, Mantripragada, & Linden, 2014). In mice, *Cacan1c* deletion was previously associated with an antidepressant phenotype (Dao et al., 2010; Kabir et al., 2016).

4.4. 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, dos Santos, Araújo, & Lopes, 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.

4.5. 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/prefrontal cortex, hippocampus, or in neurons, exclusively, and only very few authors employed similar constitutive haploinsufficiency models, such as Kabitzke 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, DuMont, & Czaja, 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, Myers, & Prescott, 2005; Kaufman et al., 2006).

5. 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 hypoactivity 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.

6. Declarations

Availability of Supporting Data and Material: Additional data and material are available from the corresponding author on reasonable request.

Competing interests: The authors declare no competing financial interests.

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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.

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General Article

GENERAL ARTICLE

Long-term environmental impact on object recognition, spatial memory and reversal learning capabilities in *Cacna1c*-haploinsufficient rats

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Abstract

Genetic and environmental influences are thought to interact in their contribution to the etiology of major neuropsychiatric disorders. One of the best replicated findings obtained in genome-wide association studies are genetic variants in the *CACNA1C* gene. Here, we used our constitutive heterozygous *Cacna1c* rat model in combination with a 4-week exposure to either post-weaning social isolation, standard housing or social and physical environmental enrichment during the critical juvenile developmental period to observe their long-term interactive effects with *Cacna1c* haploinsufficiency. Our study provides evidence for a gene × environment interaction, i.e. an interplay between *Cacna1c* haploinsufficiency and environment during juvenile development, on object recognition, spatial memory and reversal learning capabilities. Social and physical enrichment had a positive influence on *Cacna1c*$^{+/-}$ rats and *Cacna1c*$^{+/+}$ littermate controls on spatial and reversal learning, while post-weaning social isolation negatively affected novel object recognition in both genotypes. Despite intact spatial learning and re-learning abilities in all groups, slight but consistent deficits were evident in *Cacna1c*$^{+/-}$ rats previously housed under standard conditions particularly during reversal learning but not *Cacna1c*$^{+/-}$ rats previously exposed to social and physical enrichment. Together, this supports the notion that *Cacna1c* interacts with the environment to shape disease vulnerability and associated alterations in cognitive functioning.

Introduction

Genetic (16,23,72) and environmental influences (2,14) are thought to interact in their contribution to the etiology of major neuropsychiatric disorders. One of the best replicated findings obtained in genome-wide association studies (GWAS) are genetic variants in the *CACNA1C* gene (31). *CACNA1C* variants are not only implicated in affective disorders, i.e. major depressive disorder (MDD; 27) and bipolar disorder (BPD; 20), but also in

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schizophrenia (SCZ; (60)) and other neuropsychiatric disorders ((49, 84); for a recent GWAS across neuropsychiatric disorders, see (17)). Therefore, it was suggested that the *CACNA1C* gene, which encodes the voltage-dependent L-type calcium channel $Ca_v1.2$ subunit alpha-1C (8), may be considered as a cross-disorder risk factor (18).

To identify relevant communalities, research has focused on the analysis of shared endophenotypes (26), including functional and structural brain abnormalities, reduced reward sensitivity, increased stress responsivity and learning and memory impairments (29,37). While a number of studies have linked the single-nucleotide polymorphism (SNP) rs1006737 in *CACNA1C* to alterations in brain structure and function (28,61), analyses on cognitive functioning of patients and healthy participants have found conflicting results. Some found associations with lower verbal fluency (45), attention deficits (77) or impaired working memory (90) in healthy risk allele carriers of rs1006737; others found no negative impact of the risk allele on working memory (63) or overall cognitive functioning (71). In patients, the risk allele was, on the one hand, implicated in poorer speed of processing (4) and logical memory performance (32); on the other hand, no effect on cognitive functioning was found (64). An association with working memory deficits was observed in SCZ but not BPD risk allele carriers (90).

Aside from genetic components, environmental risk factors acting during critical time windows, such as childhood adversity, are known to negatively impact the development and symptom expression of MDD (14), BPD (65) and SCZ (66). Likewise, positive environmental circumstances, such as social support, seem to mitigate the risk and detrimental course of neuropsychiatric disorders (2,12,89). Importantly, environmental factors co-act or interact with genetic factors (1,5) and gene × environment (G×E) interactions were not only found to impact on neuropsychiatric diagnoses per se but also on brain structure and function (38). In fact, evidence for G×E interactions was recently also obtained in *CACNA1C* risk allele carriers, with *CACNA1C* interacting with adverse life events, such as childhood trauma, to alter the risk of developing symptoms of neuropsychiatric disorders (18) and associated brain activation patterns (44). Furthermore, in a large GWAS for systematic G×E interactions in MDD, a *CACNA1C* variant was among the reported top signals (79).

To add to the knowledge gained from correlative and quasi-experimental approaches in humans, translational animal models have been developed to investigate the function of *Cacna1c* in experimentally controlled and systematically varied designs. Through these means, *Cacna1c* has been repeatedly associated with learning and memory deficits in mice. For instance, in a first study, Moosmang *et al.* (58) showed that the inactivation of the *Cacna1c* gene in the hippocampus and neocortex led to severe impairment of hippocampus-dependent spatial memory. This effect appears to be driven by the absence of *Cacna1c* in forebrain glutamatergic neurons (18). Milder but similar phenotypes were reported by White *et al.* (83) and Temme *et al.* (75). However, no effect on cued and context fear learning was obtained in related mouse models (46,52), yet there is evidence for deficient fear extinction (76). At the neurobiological level, *Cacna1c* deletion in mice impairs hippocampal neurogenesis (47,75,82), repeatedly associated with learning and memory (59,81). Importantly, there is again support for G×E interactions. For instance, Bavley *et al.* (6) obtained evidence for a potential long-term resilience in constitutive heterozygous *Cacna1c* knockout mice exposed to chronic unpredictable stress in a spontaneous alteration working memory task. More recent findings by Dedic *et al.* (18), however, suggest that G×E interactions are depending on the time-point during development. Depletion of *Cacna1c* during early development led to increased susceptibility to chronic stress, while enhanced stress resilience was evident in adulthood. This suggests that *Cacna1c* interacts with the environment to shape disease vulnerability and associated alterations in cognitive functioning.

In the rat, *Cacna1c* heterozygosity was recently associated with impairments in reversal learning capabilities in an operant conditioning task (74) and *Cacna1c* expression in the rat hippocampus was shown to be modulated by contextual fear learning (73). In our previous studies employing *Cacna1c*-haploinsufficient rats (10), however, we did not obtain evidence for a strong association of *Cacna1c* with spatial learning deficits and effects on reversal learning were mild. However, *Cacna1c* heterozygosity leads to strong alterations in other behavioral domains, such as rough-and-tumble play and social communication through ultrasonic vocalizations (41,42). Similar to mice, *Cacna1c* haploinsufficiency was reported to be associated with reduced hippocampal cell proliferation in rats (57). Moreover, *Cacna1c* silencing in a cell culture approach was found to promote mitochondrial resilience to oxidative stress, a common cellular response to environmental stressors (55,56).

In contrast to the more commonly used homozygous models, which often elicit pronounced phenotypes but typically have weak validity with respect to neuropsychiatric disorders in humans, the analysis of heterozygous animals provides the possibility of a more moderate phenotype that can develop relevant and persistent deficits after undergoing additional hits during development through negative environmental influences, such as maltreatment (15,19), or be ameliorated in beneficial environments (59,81). As a model of maltreatment, post-weaning social isolation has proven to be effective in rodent models of neuropsychiatric disorders, inducing behavioral deficits in social communication (69) and cognitive abilities (78). Beneficial environments are often implemented as social and physical enrichment, with positive effects on various behavioral measures, including learning and memory (70).

The current study is part of a translational research effort to better understand the role of *CACNA1C* in brain structure and function (39), including a longitudinal and comprehensive deep behavioral phenotyping approach in *Cacna1c*-haploinsufficient rats. Here, we used our constitutive heterozygous *Cacna1c* rat model in combination with a 4-week exposure to either post-weaning social isolation, standard housing or social and physical environmental enrichment during the critical juvenile developmental period to observe their long-term interactive effects with *Cacna1c* haploinsufficiency on object recognition, spatial memory and reversal learning capabilities (Fig. 1).

Results

Spatial learning

Intact spatial learning abilities were evident irrespective of genotype and environment, as indicated by an effect of training day on time to criterion (D: $F_{6,180} = 28.092$; $P < 0.001$; G: $F_{1,30} = 0.256$; $P = 0.617$; D × G: $F_{6,180} = 0.580$; $P = 0.746$; E: $F_{2,30} = 1.323$; $P = 0.282$). There was, however, a significant interaction of day and experimental housing condition, with the groups previously housed in ENR displaying the fastest improvement in latency and groups previously exposed to ISO the slowest (D × E: $F_{12,180} = 2.347$; $P = 0.008$; G × E: $F_{2,30} = 0.765$; $P = 0.474$; D × G × E: $F_{12,180} = 0.352$; $P = 0.978$). When separated

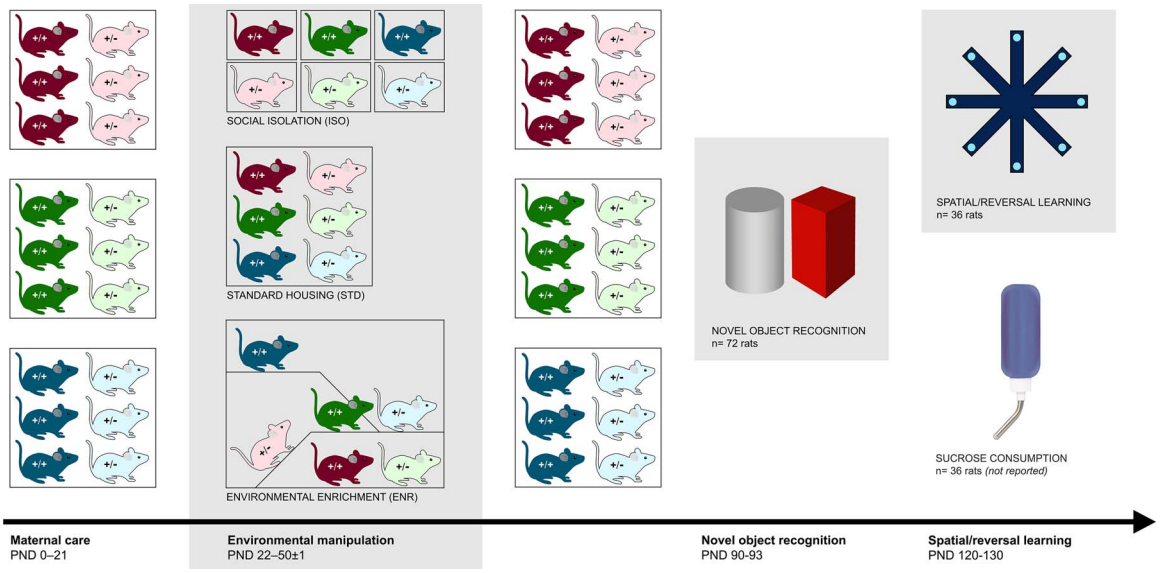

Figure 1. Timeline of testing—depiction for three exemplary litters. Male rats from N = 12 litters were housed with their mothers and siblings until PND 21. Directly after weaning on PND 21, they were housed in one of three experimental housing conditions—post-weaning social isolation (ISO), standard housing (STD) or social and physical enrichment (ENR), always in groups of six with one littermate of the opposite genotype and two matching pairs from different litters. After 4 weeks of environmental manipulation, rats were again group-housed with their littermates under standard conditions. From then on, they underwent various behavioral tests, including the novel object recognition and spatial learning and re-learning paradigms.

by genotype and environment, all groups took less time to complete the task on Day 7 than 6 days previously, with the exception of *Cacna1c*$^{+/+}$ controls previously exposed to ISO (ISO$^{+/+}$: $t_5 = 1.124$; $P = 0.156$; ISO$^{+/-}$: $t_5 = 2.784$; $P = 0.020$; STD$^{+/+}$: $t_5 = 3.045$; $P = 0.015$; STD$^{+/-}$: $t_5 = 2.733$; $P = 0.021$; ENR$^{+/+}$: $t_5 = 6.544$; $P < 0.001$; ENR$^{+/-}$: $t_5 = 3.826$; $P = 0.006$;) (Fig. 2A, left; Supplementary Material, Fig. S1A, left).

Similarly, the number of arm entries declined over time during the spatial learning phase, not modulated by genotype or housing condition (D: $F_{6,180} = 13.545$; $P < 0.001$; G: $F_{1,30} = 0.356$; $P = 0.555$; D × G: $F_{6,180} = 1.527$; $P = 0.171$; E: $F_{2,30} = 0.266$; $P = 0.768$; D × E: $F_{12,180} = 0.835$; $P = 0.614$; G × E: $F_{2,30} = 1.053$; $P = 0.361$; D × G × E: $F_{12,180} = 1.317$; $P = 0.212$). All groups entered fewer arms on Day 7 in contrast to Day 1, except for *Cacna1c*$^{+/-}$ rats previously housed in STD conditions (ISO$^{+/+}$: $t_5 = 2.152$; $P = 0.042$; ISO$^{+/-}$: $t_5 = 2.376$; $P = 0.032$; STD$^{+/+}$: $t_5 = 4.694$; $P = 0.003$; STD$^{+/-}$: $t_5 = 0.575$; $P = 0.295$; ENR$^{+/+}$: $t_5 = 3.400$; $P = 0.010$; ENR$^{+/-}$: $t_5 = 2.157$; $P = 0.042$) (Fig. 2B, left; Supplementary Material, Fig. S1B, left).

Moreover, as days of training increased, fewer erroneous arm entries occurred (D: $F_{6,180} = 22.972$; $P < 0.001$). The decrease in erroneous arm entries, however, was modulated by genotype, with *Cacna1c*$^{+/+}$ controls improving their error rate faster than *Cacna1c*$^{+/-}$ rats (G: $F_{1,30} = 0.685$; $P = 0.415$; D × G: $F_{6,180} = 3.136$; $P = 0.006$). A significant main effect of environment was also found (E: $F_{2,30} = 4.053$; $P = 0.028$; D × E: $F_{12,180} = 1.038$; $P = 0.416$; G × E: $F_{2,30} = 0.861$; $P = 0.433$; D × G × E: $F_{12,180} = 0.429$; $P = 0.950$). Post hoc analysis revealed that the groups from the ENR condition performed significantly better than the STD comparison group ($P < 0.050$). All groups made fewer wrong arm entries on Day 7 of the spatial learning period than on Day 1, excluding the group of *Cacna1c*$^{+/-}$ rats previously exposed to ISO (ISO$^{+/+}$: $t_5 = 5.843$; $P = 0.001$; ISO$^{+/-}$: $t_5 = 1.709$; $P = 0.074$; STD$^{+/+}$: $t_5 = 3.753$; $P = 0.007$; STD$^{+/-}$: $t_5 = 2.482$; $P = 0.028$; ENR$^{+/+}$: $t_5 = 4.262$; $P = 0.004$; ENR$^{+/-}$: $t_5 = 3.775$; $P = 0.007$) (Fig. 3A, left; Supplementary Material, Fig. S2A, left).

We then differentiated between different erroneous arm entries reflecting distinct memory components. Specifically, we analyzed memory errors separately for reference memory errors (RM), driven by errors in the actual long-term learning of the arm configuration, and working memory errors (WM), as a measure of short-term memory of which arms had already been entered in a specific trial. Finally, we analyzed 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, as described previously (10).

Rats made fewer entries into non-baited arms (RM errors) with increasing training days, irrespective of genotype or housing condition (D: $F_{6,180} = 7.678$; $P < 0.001$; G: $F_{1,30} = 0.969$; $P = 0.333$; D × G: $F_{6,180} = 2.000$; $P = 0.068$; E: $F_{2,30} = 1.158$; $P = 0.328$; D × E: $F_{12,180} = 1.279$; $P = 0.234$; G × E: $F_{2,30} = 1.183$; $P = 0.320$; D × G × E: $F_{12,180} = 0.569$; $P = 0.865$). A look at Day 1 vs. Day 7, however, revealed that only the ENR groups and *Cacna1c*$^{+/+}$ controls previously housed in STD conditions made fewer RM errors at the end of spatial learning, whereas there was no change in the other three groups (ISO$^{+/+}$: $t_5 = 2.381$; $P = 0.032$; ISO$^{+/-}$: $t_5 = -0.353$; $P = 0.369$; STD$^{+/+}$: $t_5 = 2.004$; $P = 0.051$; STD$^{+/-}$: $t_5 = 1.557$; $P = 0.090$; ENR$^{+/+}$: $t_5 = 2.169$; $P = 0.041$; ENR$^{+/-}$: $t_5 = 2.677$; $P = 0.022$) (Fig. 3B, left; Supplementary Material, Fig. S2B, left).

The number of repeated entries into baited arms (WM errors) also reduced over time (D: $F_{6,180} = 2.885$; $P = 0.010$; G: $F_{1,30} = 0.588$; $P = 0.449$; D × G: $F_{6,180} = 0.921$; $P = 0.481$; E: $F_{2,30} = 0.130$; $P = 0.878$; D × E: $F_{12,180} = 0.707$; $P = 0.743$; G × E: $F_{2,30} = 0.370$; $P = 0.694$; D × G × E: $F_{12,180} = 0.671$; $P = 0.778$). Yet only the two groups previously housed in ISO conditions during development made significantly fewer WM errors from Day 1 of learning to Day 7 (ISO$^{+/+}$: $t_5 = 2.887$; $P = 0.017$; ISO$^{+/-}$: $t_5 = 2.198$; $P = 0.040$; STD$^{+/+}$: $t_5 = 1.028$; $P = 0.176$; STD$^{+/-}$: $t_5 = 0.513$; $P = 0.315$; ENR$^{+/+}$: $t_5 = 1.249$; $P = 0.134$; ENR$^{+/-}$: $t_5 = 0.529$; $P = 0.310$), a pattern potentially caused by floor effects in the other groups (Fig. 4A, left; Supplementary Material, Fig. S3A, left).

For repeated entries into non-baited arms (MIX errors), an effect of training day was found and over the course of the

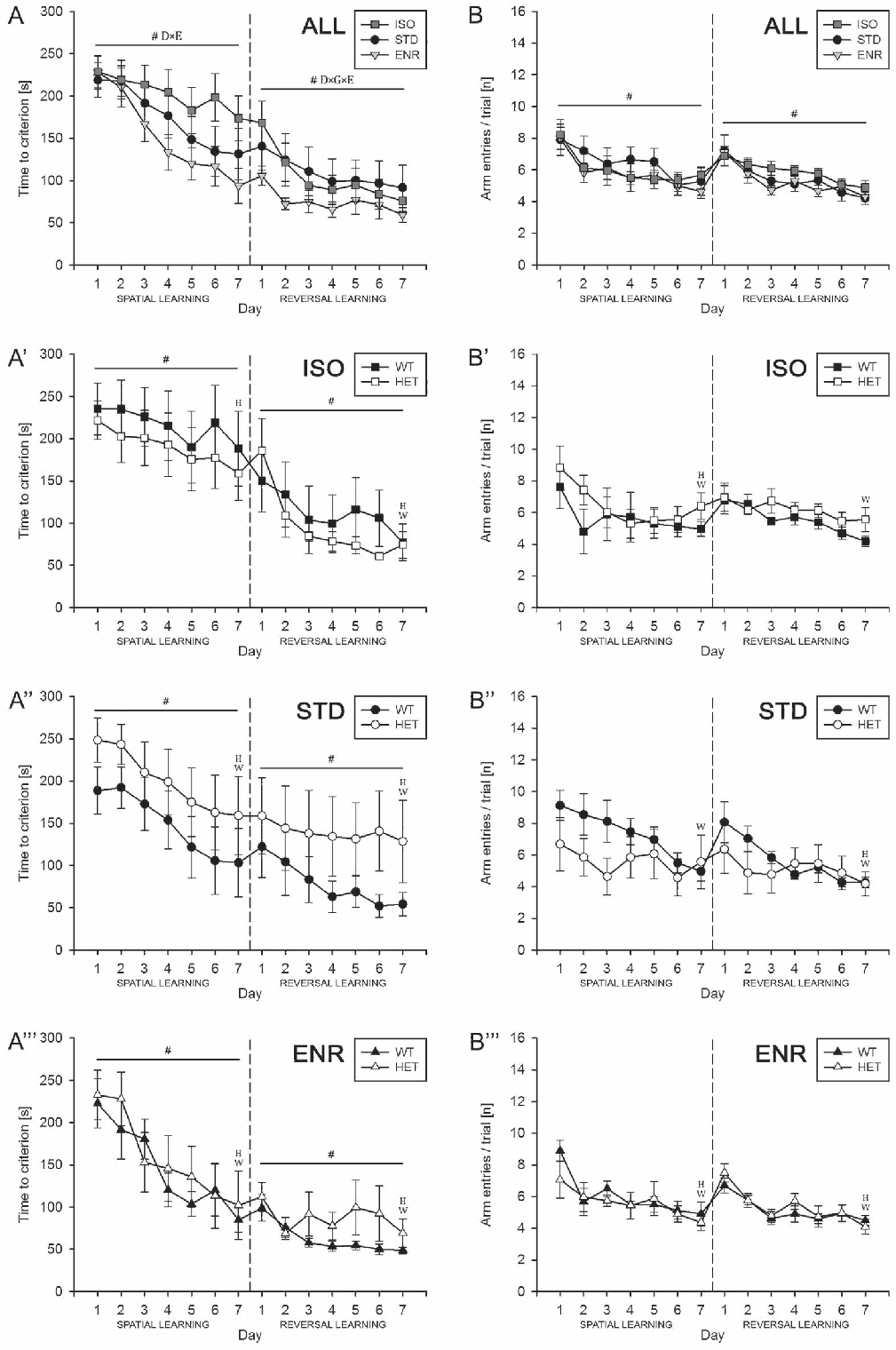

Figure 2. Spatial and reversal learning—time to criterion and arm entries. (**A**) Latency during spatial (left part) and reversal learning (right part) to collect all eight pellets per day in *Cacna1c*$^{+/+}$ and *Cacna1c*$^{+/-}$ depicted for ISO (A′), STD (A″) and ENR animals (A‴). All experimental groups became faster over days irrespective of genotype or learning phase, except for ISO$^{+/+}$ during spatial learning (A′, left). (**B**) Number of arm entries per trial until all pellets were found in *Cacna1c*$^{+/+}$ and *Cacna1c*$^{+/-}$ depicted for ISO (B′), STD (B″) and ENR animals (B‴). All experimental groups reduced the number of entries from Days 1 to 7 of both learning phases, except for STD$^{+/-}$ in spatial (B″, left) and ISO$^{+/-}$ during reversal learning (B′, right). The dashed line represents the switch from spatial to reversal learning. Time cutoff criterion was 300 s. Data are presented as means (±SEM). # = $P < 0.05$ (main effect training day). × = $P < 0.05$ (interaction effect of D = day, G = genotype, E = environment). W = $P < 0.05$ (comparison Days 1 and 7 in *Cacna1c*$^{+/+}$). H = $P < 0.05$ (comparison Days 1 and 7 in *Cacna1c*$^{+/-}$).

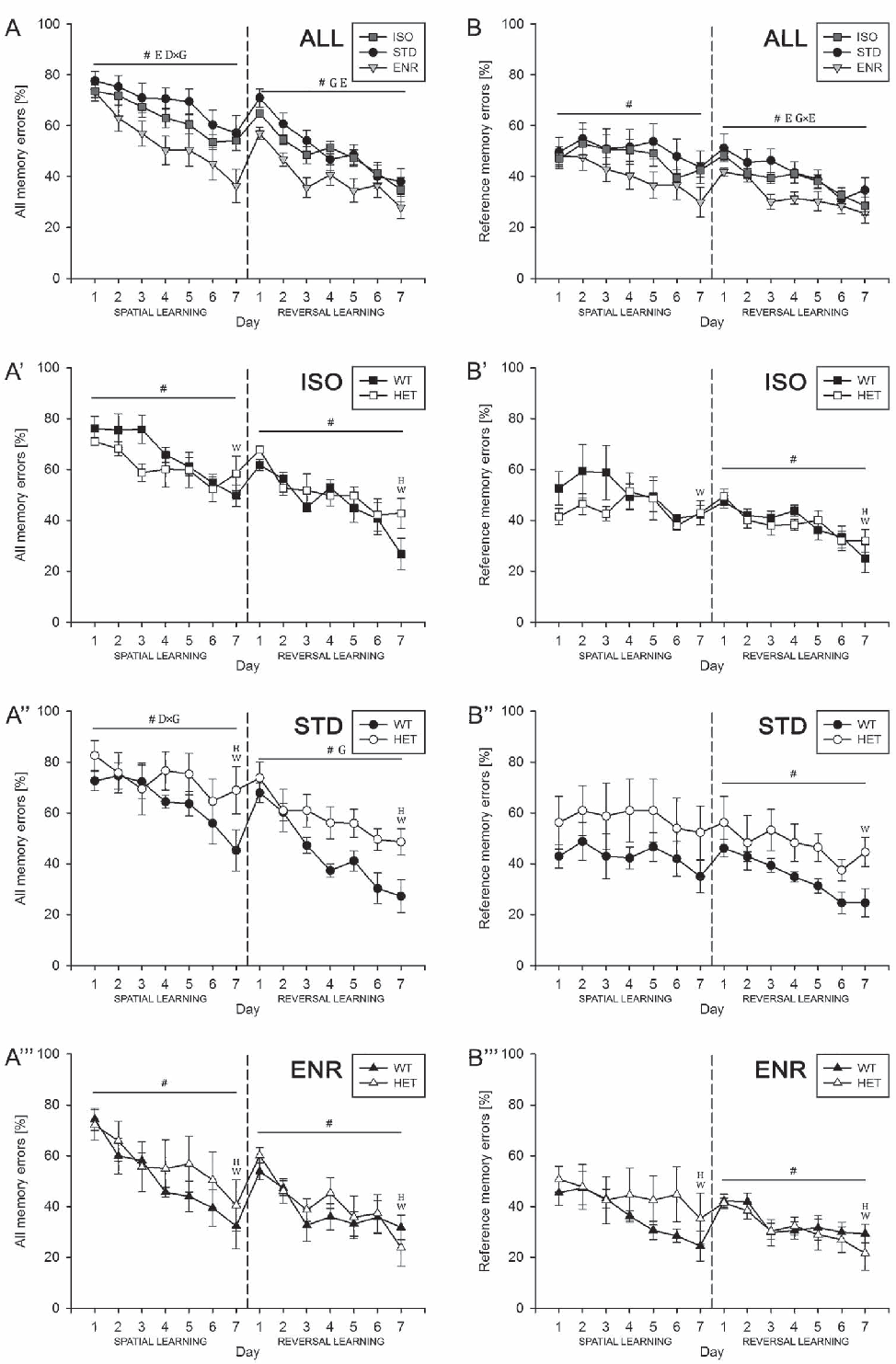

Figure 3. Spatial and reversal learning—all memory errors and reference memory errors. (**A**) Percentage of all wrong arm entries relative to total number of entries in *Cacna1c*$^{+/+}$ and *Cacna1c*$^{+/-}$ depicted for ISO (A′), STD (A″) and ENR animals (A‴). All experimental groups reduced their total error percentages from Days 1 to day 7, except for ISO$^{+/-}$ during spatial learning (A′, left). ENR condition animals displayed lower error rates in both learning phases (A‴). STD$^{+/+}$ showed steeper learning curve during spatial and better rates during reversal learning (A″, right). (**B**) Percentage of RM errors, i.e. initial entries into unrewarded arms, relative to total number of entries, in *Cacna1c*$^{+/+}$ and *Cacna1c*$^{+/-}$ depicted for ISO (B′), STD (B″) and ENR animals (B‴). Among ISO animals, *Cacna1c*$^{+/-}$ failed to improve from Days 1 to 7 of spatial learning (B′, left). There were no significant improvements in the STD condition, apart from *Cacna1c*$^{+/+}$ rats during reversal learning (B″, right). ENR animals again performed better than STD rats during reversal learning and consistently reduced number of RM errors from Days 1 to 7 (B‴). The dashed line represents the switch from spatial to reversal learning. Time cutoff criterion was 300 s. Data are presented as means (±SEM). # = $P < 0.05$ (main effect training day). G = $P < 0.05$ (main effect genotype). E = $P < 0.05$ (main effect environment). × = $P < 0.05$ (interaction effect of D = day, G = genotype, E = environment). W = $P < 0.05$ (comparison Days 1 and 7 in *Cacna1c*$^{+/+}$). H = $P < 0.05$ (comparison Days 1 and 7 in *Cacna1c*$^{+/-}$).

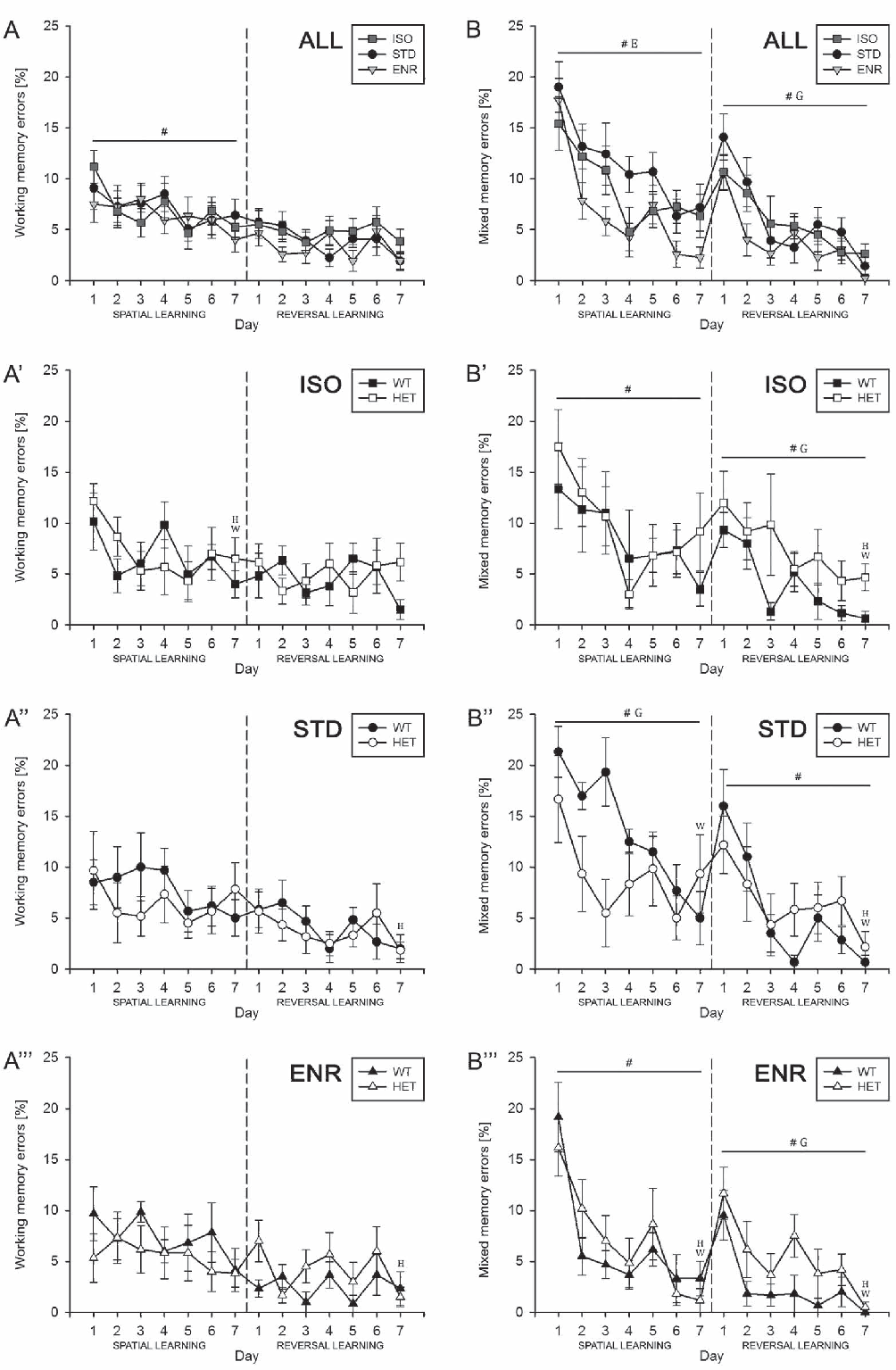

Figure 4. Spatial and reversal learning—working memory errors and mixed memory errors. (**A**) Percentage of WM errors, i.e. repeated entries into rewarded arms, relative to total number of entries in *Cacna1c*$^{+/+}$ and *Cacna1c*$^{+/-}$ depicted for ISO (A′), STD (A″) and ENR animals (A‴). A significant improvement in WM errors was achieved only by ISO groups during spatial learning (A, left), as well as *Cacna1c*$^{+/+}$ rats from the STD and ENR conditions during reversal learning (A″, right and A‴, right). (**B**) Percentage of MIX errors, i.e. repeated entries into unrewarded arms, relative to total number of entries in *Cacna1c*$^{+/+}$ and *Cacna1c*$^{+/-}$ depicted for ISO (B′), STD (B″) and ENR animals (B‴). During spatial learning, ENR animals made fewer errors than STD. Within STD, *Cacna1c*$^{+/-}$ performed better (B″, left). During reversal learning, *Cacna1c*$^{+/+}$ had lower error rates generally based on large discrepancies in ISO and ENR condition (B′, right and B‴, right). The dashed line represents the switch from spatial to reversal learning. Time cutoff criterion was 300 s. Data are presented as means (±SEM). # = $P < 0.05$ (main effect training day). G = $P < 0.05$ (main effect genotype). E = $P < 0.05$ (main effect environment). W = $P < 0.05$ (comparison Days 1 and 7 in *Cacna1c*$^{+/+}$). H = $P < 0.05$ (comparison Days 1 and 7 in *Cacna1c*$^{+/-}$).

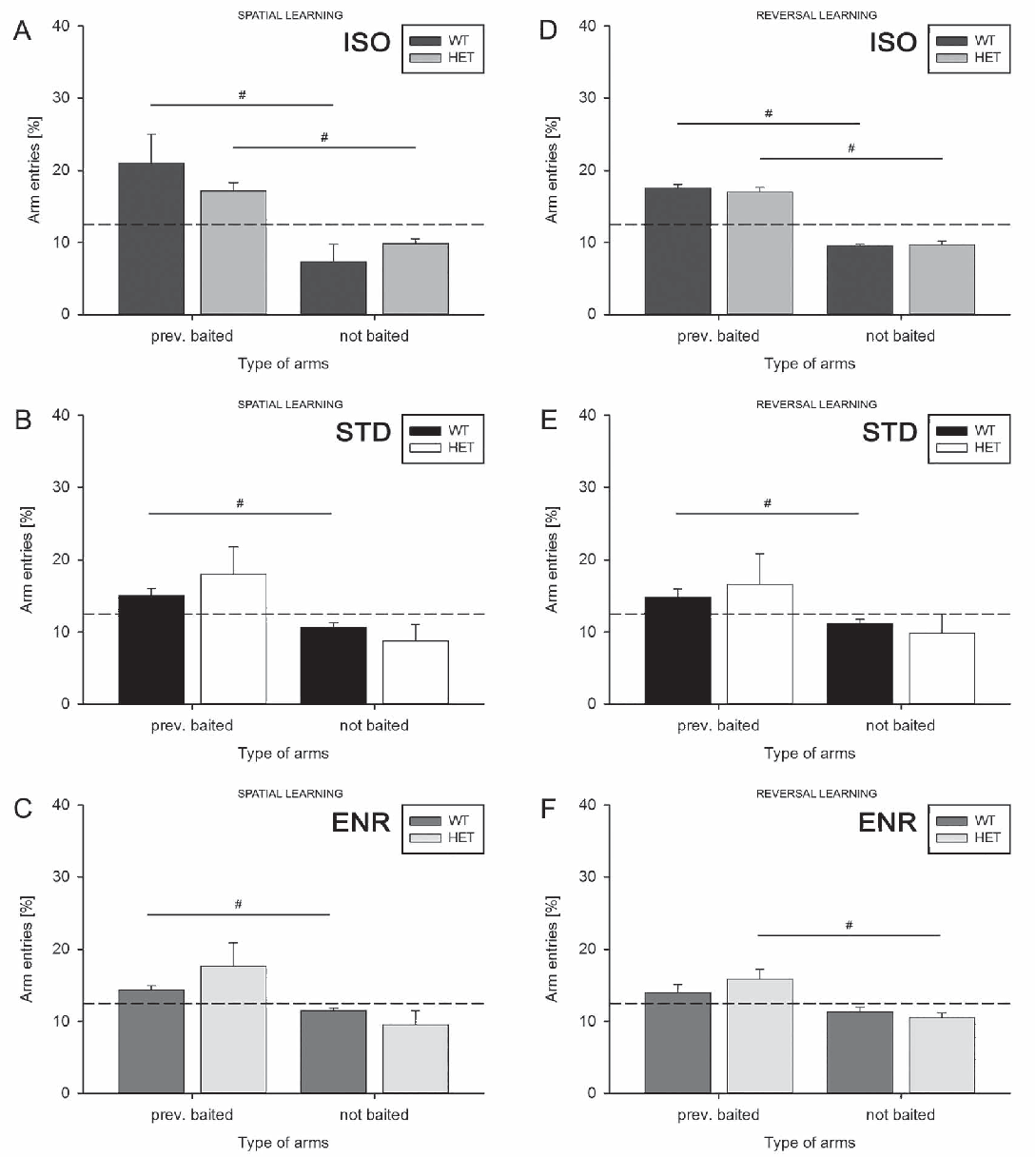

Figure 5. Spatial and reversal learning—probe trial performance. (**A–F**) Percentage of arm entries into previously baited arms relative to all probe entries and adjusted for number of arms of the specific type during spatial (A–C) and reversal learning (D–F) in *Cacna1c*$^{+/+}$ and *Cacna1c*$^{+/-}$ depicted for ISO (A, D), STD (B, E) and ENR animals (C, F). Among ISO rats, only *Cacna1c*$^{+/-}$ show a preference for previously baited arms in both probe trials, their *Cacna1c*$^{+/+}$ siblings only after reversal learning (D). In both STD and ENR conditions, no group entered the previously baited arms more than the non-baited, apart from the *Cacna1c*$^{+/+}$ groups during spatial learning (B, C). The dashed line represents chance level. Data are presented as means (±SEM). # = $P < 0.05$ (within genotype).

7-day spatial learning phase fewer MIX errors occurred irrespective of genotype (D: $F_{6,180} = 15.381$; $P < 0.001$; G: $F_{1,30} = 0.564$; $P = 0.458$; D × G: $F_{6,180} = 0.891$; $P = 0.502$), yet the decrease was dependent on housing condition (E: $F_{2,30} = 4.686$; $P = 0.017$; D × E: $F_{12,180} = 0.807$; $P = 0.643$; G × E: $F_{2,30} = 2.086$; $P = 0.142$; D × G × E: $F_{12,180} = 1.276$; $P = 0.236$). As reflected in subsequent post hoc tests, rats in the ENR condition once again proved to achieve lower error scores than STD ($P < 0.050$). In addition to the two ENR groups, only *Cacna1c*$^{+/+}$ controls previously housed in STD decreased the number of MIX errors they made from Day 1 to Day 7 (ISO$^{+/+}$: $t_5 = 2.017$; $P = 0.050$; ISO$^{+/-}$: $t_5 = 1.387$; $P = 0.112$; STD$^{+/+}$: $t_5 = 4.557$; $P = 0.003$; STD$^{+/-}$: $t_5 = 1.313$; $P = 0.123$; ENR$^{+/+}$: $t_5 = 6.181$; $P = 0.001$; ENR$^{+/-}$: $t_5 = 4.306$; $P = 0.004$) (Fig. 4B, left; Supplementary Material, Fig. S3B, left).

In the following probe trial, all *Cacna1c*$^{+/+}$ controls distinguished successfully between previously rewarded and non-rewarded arms (ISO$^{+/+}$: $t_5 = 2.133$; $P = 0.043$; STD$^{+/+}$: $t_5 = 2.632$; $P = 0.023$; ENR$^{+/+}$: $t_5 = 2.996$; $P = 0.015$), whereas only *Cacna1c*$^{+/-}$ rats previously exposed to ISO preferred the rewarded arms to the non-rewarded arms (ISO$^{+/-}$: $t_5 = 3.990$; $P = 0.005$; STD$^{+/-}$: $t_4 = 1.532$; $P = 0.100$; ENR$^{+/-}$: $t_5 = 1.575$; $P = 0.080$) (Fig. 5A–C). Neither genotype nor housing conditions influenced the proportion of entries made into the previously rewarded arms (G: $F_{1,30} = 0.092$; $P = 0.764$; E: $F_{2,30} = 1.041$; $P = 0.366$; G×E: $F_{2,30} = 1.611$; $P = 0.217$), or into the non-rewarded arms (G: $F_{1,30} = 0.045$; $P = 0.834$; E: $F_{2,30} = 0.869$; $P = 0.430$; G×E: $F_{2,30} = 1.763$; $P = 0.189$). Of note, body weight during spatial learning was not affected by genotype or housing conditions but slightly decreased from Day

1 to Day 7 by ~6 grams from ~382 to ~376 g (D: $F_{6,180} = 21.620$; $P < 0.001$; G: $F_{1,30} = 0.568$; $P = 0.457$; D×G: $F_{6,180} = 1.111$; $P = 0.358$; E: $F_{2,30} = 0.145$; $P = 0.865$; D×E: $F_{12,180} = 0.532$; $P = 0.892$; G×E: $F_{2,30} = 1.006$; $P = 0.378$; D×G×E: $F_{12,180} = 0.787$; $P = 0.664$).

Reversal learning

During reversal learning, too, time to criterion reduced over days, reflecting intact reversal learning abilities (D: $F_{6,180} = 20.444$; $P < 0.001$). Importantly, however, a G×E interaction with training day uncovered a more prominent genotype difference in the STD housing condition with slower *Cacna1c*$^{+/-}$ rats, in contrast to *Cacna1c*$^{+/+}$ controls (G: $F_{1,30} = 1.048$; $P = 0.314$; D×G: $F_{6,180} = 1.057$; $P = 0.390$; E: $F_{2,30} = 0.883$; $P = 0.424$; D×E: $F_{12,180} = 1.799$; $P = 0.051$; G×E: $F_{2,30} = 1.015$; $P = 0.374$; D×G×E: $F_{12,180} = 2.282$; $P = 0.010$). All six groups were faster on Day 7 in contrast to Day 1 (ISO$^{+/+}$: $t_5 = 4.182$; $P = 0.005$; ISO$^{+/-}$: $t_5 = 3.327$; $P = 0.011$; STD$^{+/+}$: $t_5 = 2.831$; $P = 0.019$; STD$^{+/-}$: $t_5 = 4.068$; $P = 0.005$; ENR$^{+/+}$: $t_5 = 3.413$; $P = 0.010$; ENR$^{+/-}$: $t_5 = 4.561$; $P = 0.003$) (Fig. 2A, right; Supplementary Material, Fig. S1A, right).

Intact reversal learning was also reflected by an effect of training day on arms entered until trial completion (D: $F_{6,180} = 16.799$; $P < 0.001$; G: $F_{1,30} = 0.079$; $P = 0.781$; D×G: $F_{6,180} = 1.533$; $P = 0.170$; E: $F_{2,30} = 0.770$; $P = 0.472$; D×E: $F_{12,180} = 0.783$; $P = 0.667$; G × E: $F_{2,30} = 0.618$; $P = 0.546$; D×G×E: $F_{12,180} = 1.088$; $P = 0.373$). This reduction of arm entries was also apparent in all groups but isolated *Cacna1c*$^{+/-}$ rats between the last day of reversal learning and on Day 1 (ISO$^{+/+}$: $t_5 = 2.553$; $P = 0.026$; ISO$^{+/-}$: $t_5 = 1.662$; $P = 0.079$; STD$^{+/+}$: $t_5 = 2.669$; $P = 0.022$; STD$^{+/-}$: $t_5 = 2.491$; $P = 0.028$; ENR$^{+/+}$: $t_5 = 4.491$; $P = 0.003$; ENR$^{+/-}$: $t_5 = 4.880$; $P = 0.003$) (Fig. 2B, right; Supplementary Material, Fig. S1B, right).

With increasing days of reversal training, fewer errors occurred (D: $F_{6,180} = 30.688$; $P < 0.001$). Importantly, *Cacna1c*$^{+/+}$ controls were, in general, less prone to erroneous entries than their *Cacna1c*$^{+/-}$ littermates (G: $F_{1,30} = 6.461$; $P = 0.016$; D×G: $F_{6,180} = 1.069$; $P = 0.383$). Moreover, the number of erroneous arm entries was affected by housing condition (E: $F_{2,30} = 7.140$; $P = 0.003$; D×E: $F_{12,180} = 0.926$; $P = 0.522$; G×E: $F_{2,30} = 1.755$; $P = 0.190$; D×G×E: $F_{12,180} = 1.216$; $P = 0.275$). As revealed by post hoc analysis, rats previously housed in ENR conditions made fewer errors than rats in both of the two other housing conditions ($P < 0.050$). The reduction of all errors from Day 1 to Day 7 was significant for all groups (ISO$^{+/+}$: $t_5 = 5.371$; $P = 0.002$; ISO$^{+/-}$: $t_5 = 3.531$; $P = 0.009$; STD$^{+/+}$: $t_5 = 5.216$; $P = 0.002$; STD$^{+/-}$: $t_5 = 4.618$; $P = 0.003$; ENR$^{+/+}$: $t_5 = 4.081$; $P = 0.005$; ENR$^{+/-}$: $t_5 = 4.694$; $P = 0.003$) (Fig. 3A, right; Supplementary Material, Fig. S2A, right).

Consistently, fewer RM errors occurred over reversal training days (D: $F_{6,180} = 16.080$; $P < 0.001$). Again, however, there was evidence for a G×E interaction and the divergence between genotypes was higher in previously STD-housed groups, with *Cacna1c*$^{+/+}$ controls performing better than their *Cacna1c*$^{+/-}$ littermates (G: $F_{1,30} = 2.014$; $P = 0.166$; D×G: $F_{6,180} = 0.449$; $P = 0.845$; G×E: $F_{2,30} = 3.350$; $P = 0.049$). This deficit of the heterozygous genotype was less apparent in animals previously exposed to ENR and ISO where both genotypes were on par with each other. Moreover, the number of RM errors was affected by housing condition (E: $F_{2,30} = 4.013$; $P = 0.029$; D×E: $F_{12,180} = 0.912$; $P = 0.536$; D×G×E: $F_{12,180} = 0.627$; $P = 0.818$). Post hoc tests indicated that once again, animals previously exposed to ENR housing conditions had better reference memory than animals previously housed in STD ($P < 0.050$). All groups improved from Day 1 to the last spatial learning day (ISO$^{+/+}$: $t_5 = 5.163$; $P = 0.002$; ISO$^{+/-}$: $t_5 = 2.770$; $P = 0.020$; STD$^{+/+}$: $t_5 = 3.948$; $P = 0.006$; STD$^{+/-}$: $t_5 = 1.507$; $P = 0.096$; ENR$^{+/+}$: $t_5 = 3.034$; $P = 0.015$; ENR$^{+/-}$: $t_5 = 2.529$; $P = 0.027$) with the exception of *Cacna1c*$^{+/-}$ rats previously housed in STD (Fig. 3B, right; Supplementary Material, Fig. S2B, right).

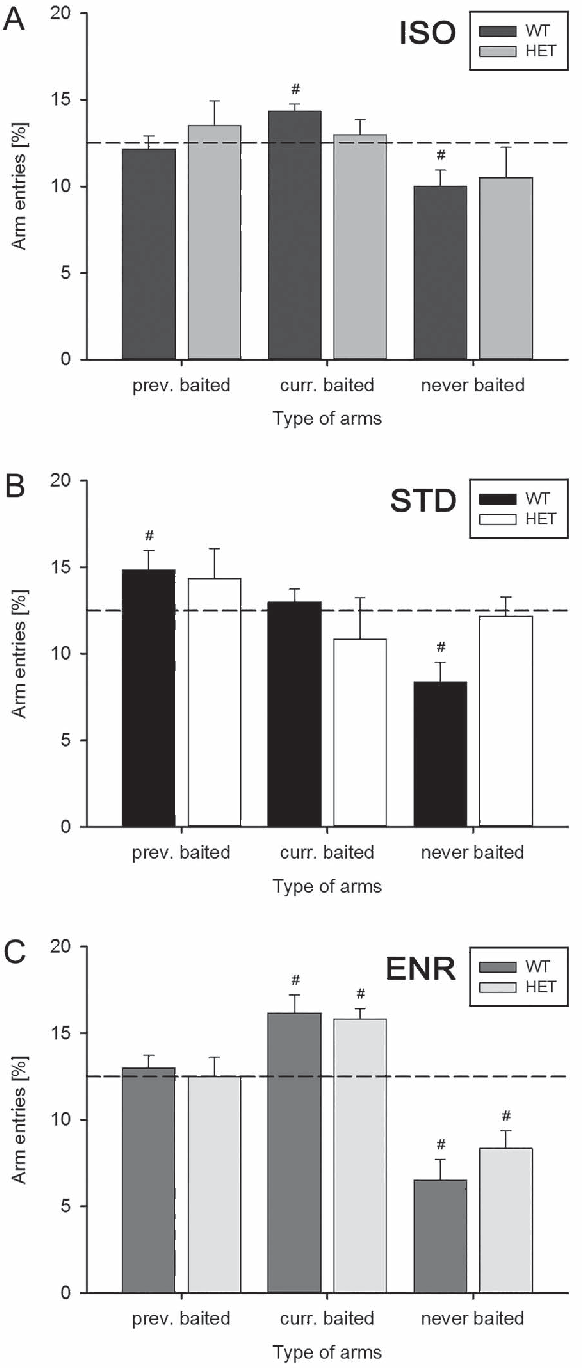

Figure 6. Spatial and reversal learning—cognitive flexibility. (**A–C**) Percentage of entries made into previously rewarded arms from spatial learning (*prev.*), currently rewarded arms from this day forward (*curr.*) and the two never baited arms depicted for ISO (A), STD (B) and ENR animals (C). Only *Cacna1c*$^{+/+}$ rats entered the new arms above chance level among ISO animals (A). In STD housing conditions, *Cacna1c*$^{+/+}$ still showed a preference for the no longer relevant arms (B), whereas both enriched *Cacna1c*$^{+/+}$ and *Cacna1c*$^{+/-}$ adapt quickly to the new reward locations (C). The dashed line represents chance level. Data are presented as means (±SEM). # = $P < 0.05$ (within genotype, comparison to chance level).

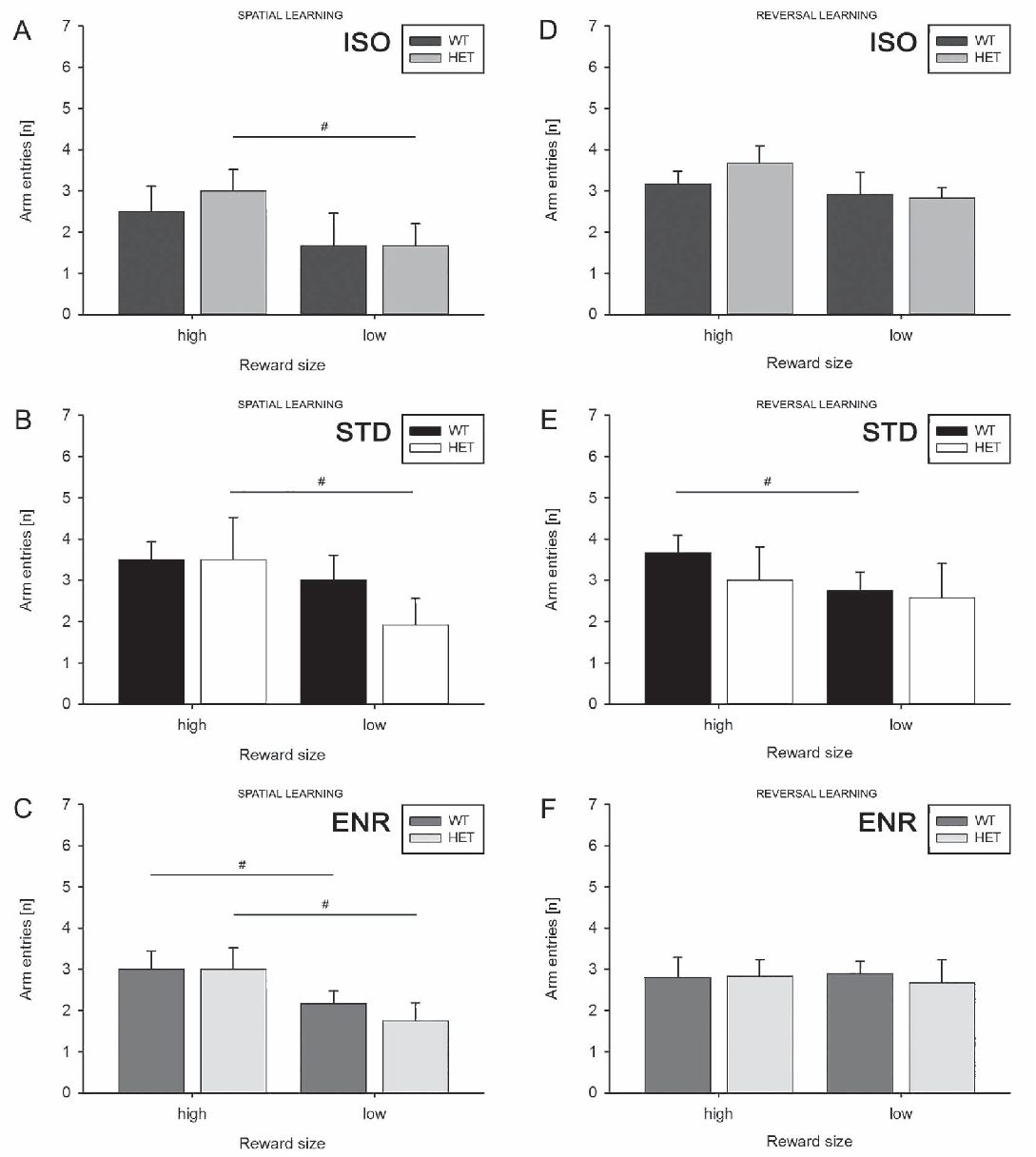

Figure 7. Spatial and reversal learning—reward sensitivity. (**A–F**) Number of entries made into the arm previously containing six pellets (*high*) and arms with one pellet during learning (*low*, average of two arms) during spatial learning probe trial (A–C) and reversal learning probe trial (D–F) depicted for ISO (A, D), STD (B, E) and ENR animals (C, F). Only *Cacna1c*$^{+/-}$ groups preferred the higher rewarded arm over the lower rewarded arms during the spatial learning probe trial (A–C). In the reversal learning probe trial, only *Cacna1c*$^{+/+}$ in STD housing showed a preference (E). The dashed line represents chance level. Data are presented as means (±SEM). # = $P < 0.05$ (within genotype).

No progress was observed for WM errors, as repeated entries into rewarded arms did not decrease over training days (D: $F_{6,180} = 2.090$; $P = 0.056$; G: $F_{1,30} = 0.770$; $P = 0.387$; D×G: $F_{6,180} = 1.593$; $P = 0.151$; E: $F_{2,30} = 1.407$; $P = 0.260$; D×E: $F_{12,180} = 0.776$; $P = 0.675$; G×E: $F_{2,30} = 0.700$; $P = 0.504$; D×G×E: $F_{12,180} = 0.860$; $P = 0.589$), and no group apart from *Cacna1c*$^{+/-}$ rats from STD and ENR housing improved from the first to the last day of training (ISO$^{+/+}$: $t_5 = 1.363$; $P = 0.116$; ISO$^{+/-}$: $t_5 = 0.000$; $P = 0.500$; STD$^{+/+}$: $t_5 = 1.295$; $P = 0.126$; STD$^{+/-}$: $t_5 = 2.112$; $P = 0.044$; ENR$^{+/+}$: $t_5 = 0.000$; $P = 0.500$; ENR$^{+/-}$: $t_5 = 2.356$; $P = 0.033$) (Fig. 4A, right; Supplementary Material, Fig. S3A, right).

Conversely, MIX error performance improved, in that repeated entries into unrewarded arms were made more rarely as training days increased (D: $F_{6,180} = 15.642$; $P < 0.001$). As with general error count, *Cacna1c*$^{+/+}$ controls performed better than their *Cacna1c*$^{+/-}$ counterparts (G: $F_{1,30} = 6.866$; $P = 0.014$; D×G: $F_{6,180} = 0.620$; $P = 0.714$; E: $F_{2,30} = 2.154$; $P = 0.134$; D×E: $F_{12,180} = 0.944$; $P = 0.504$; G×E: $F_{2,30} = 0.761$; $P = 0.476$; D×G×E: $F_{12,180} = 0.855$; $P = 0.594$). All groups performed well in the comparison between Day 7 and Day 1 of reversal learning (ISO$^{+/+}$: $t_5 = 4.174$; $P = 0.005$; ISO$^{+/-}$: $t_5 = 2.284$; $P = 0.036$; STD$^{+/+}$: $t_5 = 4.072$; $P = 0.005$; STD$^{+/-}$: $t_5 = 3.932$; $P = 0.006$; ENR$^{+/+}$: $t_5 = 3.906$; $P = 0.006$; ENR$^{+/-}$: $t_5 = 3.958$; $P = 0.006$) (Fig. 4B, right; Supplementary Material, Fig. S3B, right).

During the probe trial at the end of the last reversal learning day, all previously isolated animals displayed a preference for the

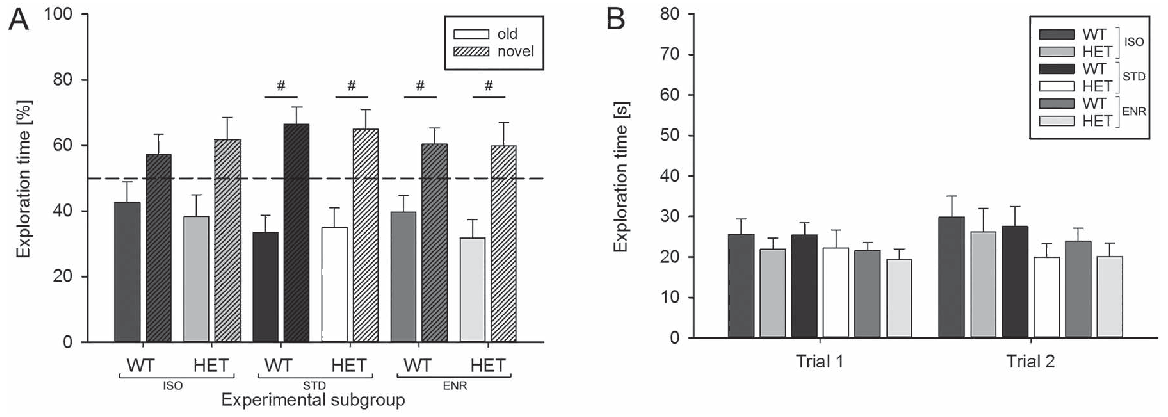

Figure 8. Novel object recognition—distinction and exploration. (**A**) Time spent sniffing (percentage of total exploration in Trial 2) the novel object (patterned bars) vs. the familiar object (blank bars). STD and ENR housed groups could distinguish between novel and old objects; preferring to sniff the novel object for longer, ISO animals failed to make the distinction. (**B**) Total time spent exploring in both trials. There was no difference between groups in seconds spent exploring in Trial 1 or Trial 2. The dashed line represents chance level. Data are presented as means (±SEM). # = $P < 0.05$ (within genotype).

previously rewarded arms ($ISO^{+/+}$: $t_5 = 10.328$; $P < 0.001$; $ISO^{+/-}$: $t_5 = 6.574$; $P < 0.001$). Within animals previously housed in STD conditions, only *Cacna1c*$^{+/+}$ controls entered the rewarded arms more frequently than the non-rewarded arms ($STD^{+/+}$: $t_5 = 2.149$; $P = 0.042$), just as enriched *Cacna1c*$^{+/-}$ rats ($ENR^{+/-}$: $t_5 = 2.549$; $P = 0.026$). The other two groups failed to make the distinction ($STD^{+/-}$: $t_5 = 0.960$; $P = 0.191$; $ENR^{+/+}$: $t_4 = 2.025$; $P = 0.057$) (Fig. 5D–F). For the proportion of entries made into either arm type, neither the genotype nor the environmental manipulation the animals had undergone made a difference (previously rewarded: G: $F_{1,30} = 0.229$; $P = 0.636$; E: $F_{2,30} = 0.547$; $P = 0.585$; G×E: $F_{2,30} = 0.161$; $P = 0.852$ and non-rewarded: G: $F_{1,30} = 0.314$; $P = 0.579$; E: $F_{2,30} = 0.515$; $P = 0.603$; G×E: $F_{2,30} = 0.198$; $P = 0.821$). Of note, body weight during reversal learning was not affected by genotype or housing conditions and the decrease from Day 1 to Day 7 was very mild with ∼2 g from ∼380 to ∼378 g (D: $F_{6,180} = 1.363$; $P = 0.232$; G: $F_{1,30} = 0.524$; $P = 0.475$; D×G: $F_{6,180} = 0.233$; $P = 0.965$; E: $F_{2,30} = 0.057$; $P = 0.945$; D×E: $F_{12,180} = 1.255$; $P = 0.249$; G×E: $F_{2,30} = 1.041$; $P = 0.366$; D×G×E: $F_{12,180} = 0.808$; $P = 0.642$).

Cognitive flexibility

On the first day of reversal learning, an additional measure of cognitive flexibility was taken by comparing proportional entries made into the arms previously rewarded during spatial learning ('previous'), the ones currently and newly rewarded during reversal learning ('current') and those two arms that never once contained bait ('never') to chance level (12.5%), as previously described (10). In *Cacna1c*$^{+/+}$ controls previously housed in ISO, a pattern indicative of intact cognitive flexibility was found, in that they entered the previously baited arms at chance level, the newly baited arms above chance level and the never baited arms below chance level ($ISO^{+/+}$: previous: $t_5 = -0.445$; $P = 0.338$; current: $t_5 = 4.348$; $P = 0.004$; never: $t_5 = -2.685$; $P = 0.022$). Their *Cacna1c*$^{+/-}$ counterparts, on the other hand, entered all arm types at chance level, showing no preference for any arm type ($ISO^{+/-}$: previous: $t_5 = 0.698$; $P = 0.258$; current: $t_5 = 0.584$; $P = 0.293$; never: $t_5 = -1.145$; $P = 0.152$) (Fig. 6A). A similar result was obtained for *Cacna1c*$^{+/-}$ rats previously housed in standard conditions ($STD^{+/-}$: previous: $t_5 = 1.039$; $P = 0.173$; current: $t_5 = -0.690$; $P = 0.261$; never: $t_5 = -0.293$; $P = 0.391$). *Cacna1c*$^{+/+}$ controls previously housed in STD demonstrated a continued preference for the previously rewarded, no longer relevant arms ($STD^{+/+}$: previous: $t_5 = 2.051$; $P = 0.048$; current: $t_5 = 0.685$; $P = 0.262$; never: $t_5 = -3.550$; $P = 0.008$), suggesting perseveration tendencies (Fig. 6B). Contrarily, both genotypes in the enriched housing condition displayed a predilection for the currently rewarded arms on the first day of reversal learning ($ENR^{+/+}$: previous: $t_5 = 0.685$; $P = 0.262$; current: $t_5 = 3.505$; $P = 0.009$; never: $t_5 = -4.983$; $P = 0.002$ and $ENR^{+/-}$: previous: $t_5 = 0.000$; $P = 0.500$; current: $t_5 = 5.547$; $P = 0.002$; never: $t_5 = -4.077$; $P = 0.005$) (Fig. 6C), a sign of high cognitive flexibility.

Reward sensitivity

To assess reward sensitivity, probe trial entries into the highly rewarded arm (six pellets) were compared to entries into the less rewarded arm (one pellet each, mean of two arms), as previously described (10). During the spatial learning probe, a clear preference for the highly rewarded arm was found for all *Cacna1c*$^{+/-}$ rats, regardless of housing condition ($ISO^{+/-}$: $t_5 = 4.339$; $P = 0.004$; $STD^{+/-}$: $t_5 = 3.997$; $P = 0.005$; $ENR^{+/-}$: $t_5 = 7.319$; $P < 0.001$), whereas among the *Cacna1c*$^{+/+}$ control groups only the enriched animals displayed a higher affinity towards the highly rewarded arm ($ISO^{+/+}$: $t_5 = 1.206$; $P = 0.141$; $STD^{+/+}$: $t_5 = 1.168$; $P = 0.148$; $ENR^{+/+}$: $t_5 = 2.076$; $P = 0.047$) (Fig. 7A–C).

In the reversal learning trial, preference for the highly rewarded arm was only apparent in the *Cacna1c*$^{+/+}$ controls previously housed under STD conditions ($STD^{+/+}$: $t_5 = 11.000$; $P < 0.001$), while none of the other five groups distinguished between both arm types ($STD^{+/-}$: $t_5 = 1.746$; $P = 0.071$; $ISO^{+/+}$: $t_5 = 0.889$; $P = 0.208$; $ISO^{+/-}$: $t_5 = 1.890$; $P = 0.059$; $ENR^{+/+}$: $t_4 = -0.232$; $P = 0.414$; $ENR^{+/-}$: $t_5 = 0.466$; $P = 0.331$) (Fig. 7D–F).

Novel object recognition

Rats previously exposed to ISO did not show a preference for the novel object, irrespective of genotype ($ISO^{+/-}$: $t_{11} = 1.740$; $P = 0.055$; $ISO^{+/+}$: $t_{11} = 1.173$; $P = 0.133$). All other four groups distinguished well between old and novel objects ($STD^{+/+}$: $t_{11} = 3.163$; $P = 0.005$; $STD^{+/-}$: $t_{11} = 2.497$; $P = 0.015$; $ENR^{+/+}$: $t_{11} = 2.053$; $P = 0.033$; $ENR^{+/-}$: $t_{11} = 2.885$; $P = 0.008$) (Fig. 8A). There was no difference in general exploration between genotypes or housing conditions in either Trial 1 (G: $F_{1,72} = 1.340$; $P = 0.251$; E: $F_{2,72} = 0.715$; $P = 0.493$; G×E: $F_{2,72} = 0.028$; $P = 0.973$) or Trial

2 (G: $F_{1,72} = 1.968$; $P = 0.165$; E: $F_{2,72} = 1.003$; $P = 0.372$; G×E: $F_{2,72} = 0.131$; $P = 0.878$) (Fig. 8B). Of note, body weight during novel object recognition was not affected (G: $F_{1,72} = 0.733$; $P = 0.395$; E: $F_{2,72} = 0.179$; $P = 0.837$; G×E: $F_{2,72} = 0.289$; $P = 0.750$).

Discussion

The aim of this study was to investigate the long-term impact of environmental factors, in the form of post-weaning social isolation or social and physical enrichment, in contrast to standard housing conditions, on spatial memory, reversal learning and object recognition in a constitutive heterozygous *Cacna1c* rat model. For this purpose, we employed a radial arm maze learning task and the novel object recognition paradigm in adult rats housed in groups under standard conditions several weeks after manipulating their environment as juveniles. Social and physical enrichment had a positive influence on *Cacna1c*$^{+/-}$ rats and *Cacna1c*$^{+/+}$ littermate controls on spatial and reversal learning, while post-weaning social isolation negatively affected novel object recognition in both genotypes. Despite intact spatial learning and re-learning abilities in all groups, slight but consistent deficits were evident in *Cacna1c*$^{+/-}$ rats previously housed under standard conditions particularly during reversal learning but not *Cacna1c*$^{+/-}$ rats previously exposed to social and physical enrichment.

Effects of *Cacna1c* haploinsufficiency on novel object recognition

In line with our previous *Cacna1c* rat studies (10), *Cacna1c* haploinsufficiency did not affect object recognition memory. This is also consistent with available mouse studies (18;34,88), suggesting that basal cognitive functioning is intact.

Effects of *Cacna1c* haploinsufficiency on spatial learning and working memory

Cacna1c haploinsufficiency also had no major impact on spatial learning and working memory, with both *Cacna1c*$^{+/-}$ rats and *Cacna1c*$^{+/+}$ controls displaying intact spatial learning capabilities in the absence of noteworthy deficits in reference and working memory, yet minor deficits in probe trial performance. Intact spatial learning was reflected in a reduced time to criterion, a decrease in the number of arm entries and a decreased error count, including reference, working and mixed memory errors, although the decrease in error count was weaker in *Cacna1c*$^{+/-}$ rats than *Cacna1c*$^{+/+}$ controls. There was also a preference for the previously rewarded arms in the spatial learning probe trial, yet again this preference was less prominent in *Cacna1c*-haploinsufficient rats.

These present observations resonate well with our previous *Cacna1c* rat studies (10), yet they do not mirror most of the literature on *Cacna1c* mouse models. Moosmang *et al.* (58) showed that the inactivation of the *Cacna1c* gene in the hippocampus and neocortex of mice led to severe impairment of hippocampus-dependent spatial memory, with no continuous improvement after the second training day. Moreover, a general superiority of wild-type to *Cacna1c* knockout mice was indicated in the display of more effective search strategies in a labyrinth maze task, although no impairments during two probe trials were found (58). Milder but similar phenotypes were reported by White *et al.* (83) and Temme *et al.* (75). White *et al.* (83) reported latency decreases for both wild-type and *Cacna1c* knockout mice in the Morris water maze (MWM) and equal performance of both genotypes in the immediately following probe trial. However, while they did not observe initial probe trial differences, pronounced impairments in conditional prefrontal cortex knockouts were evident in a probe trial 30 days after learning. This begs the question of whether the differences we observed would also be more obvious if testing for long-term memory. Another variable that appears to yield differential results is task difficulty, as shown by Temme *et al.* (75), who found no genotype effects in a regular MWM task, but major difficulties in *Cacna1c* knockouts in a limited cue version of the test. However, in line with the lack of prominent spatial learning deficits in our *Cacna1c* rat model is the fact that no effect on cued and context fear learning was obtained in related mouse models (46,52). Also, in a procedural T-maze task, heterozygous *Cacna1c* mice reached success criteria at similar rates as wild-type controls within 5–7 days of training (36), although it has to be noted that this task requires egocentric navigation more than the usage of spatial cues. Importantly, a differential effect of knockout timing was found by Dedic *et al.* (18). When examining mice that underwent homozygous deletion of *Cacna1c* from forebrain glutamatergic neurons in adulthood, they found intact learning abilities in a water cross maze task even after 30 days had passed, yet if *Cacna1c* was inactivated during early development the animals barely surpassed chance levels in their correct choice rates.

No specific working memory deficits were evident in our study, a finding concurrent with a study of *Cacna1c*-haploinsufficient mice (88). Just as in our previous study (10), the authors found no genotype effect on working memory performance in a Y-maze paradigm (88).

Effects of *Cacna1c* haploinsufficiency on reversal learning and cognitive flexibility

Cacna1c-haploinsufficient rats further displayed intact reversal learning capabilities, as reflected in a reduced time to criterion, a decrease in the number of arm entries and a decreased error count. However, the reduction in erroneous entries over the second 7-day learning phase was clearly less prominent in *Cacna1c*$^{+/-}$ rats than in *Cacna1c*$^{+/+}$ controls. While *Cacna1c* haploinsufficiency again did not affect working memory performance, particularly mixed memory errors occurred more often in *Cacna1c*$^{+/-}$ rats than in *Cacna1c*$^{+/+}$ controls. There was no major impact of *Cacna1c* haploinsufficiency on cognitive flexibility and the preference for the previously rewarded arms in the reversal learning probe trial.

Regarding genotype comparisons, this is in accordance with our previous *Cacna1c* study (10). More pronounced genotype differences were found in a recent study by Sykes *et al.* (74), where heterozygous *Cacna1c* rats made more errors than the wild-type comparison group during reversal learning, even though they were on par during the visual discrimination required before, indicating that any observed memory deficits are probably not based on visual impairments in the *Cacna1c*$^{+/-}$ genotype. Moreover, there is evidence for fear extinction deficits (76). Similar performance of both genotypes, however, was observed in the reversal phase of the procedural T-maze in mice (36). Importantly, as for spatial learning, Dedic *et al.* (18) again obtained evidence for strong developmental effects. Mice exposed to homozygous deletion of *Cacna1c* from forebrain glutamatergic neurons during early development barely performed better than at chance level during water cross maze reversal, while their adult counterparts even displayed enhanced cognitive flexibility.

Environmental impact on object recognition, spatial memory and reversal learning capabilities

Post-weaning social isolation exhibited a significant negative influence on novel object recognition in both *Cacna1c*$^{+/-}$ rats and *Cacna1c*$^{+/+}$ controls that appeared unable to distinguish the novel object from the familiar one during the test trial, even though exploration levels were on par with the animals from other environmental conditions. These results are broadly consistent with the ones obtained in rats by McIntosh *et al.* (51), who found no difference in exploratory behavior but impaired object recognition in rats isolated after weaning. Similar evidence comes from Bianchi *et al.* (9)). It has to be said, though, that both of the aforementioned studies tested the rats directly after the environmental housing manipulation. Evidence that consistent long-term impairments follow temporary social isolation after weaning was obtained in the current experiment, as well as in a previous study conducted in our lab that tested animals with a very similar delay of 6 weeks after environmental manipulation (78). Fischer *et al.* (21) furthermore showed that this negative effect of isolation can be found in rats even if the environmental manipulation occurs later in life, although sex-specific effects might play a modulatory role (53).

Social and physical enrichment did not appear to affect novel object recognition memory much, as shown in various experiments performed on rats and mice (13,54,67), although enriched rats did show a stronger preference than standard housed animals after inter-trial intervals of more than 1 h (13;54), as well as a longer time spent in enriched housing (33). A shorter time between acquisition and recall, on the other hand, caused enriched rats to show even less of a bias towards the novel object (67). In our animals, exposure to social isolation during development had by far the most prominent effect on novel object recognition abilities, in contrast to environmental enrichment and genotype influences.

The literature on the effect of social isolation on spatial and reversal learning is mixed. Some studies have found isolation not to cause a specific change in Y-maze performance in mice (62) or radial arm maze learning abilities in rats (68), while others found animals to be retarded in the acquisition of a rotational T-maze task (50) and non-spatial cognitive flexibility measures (3,35) following several weeks of isolation housing. Still others observed enhanced spatial and reversal learning (85). No consistent effect of post-weaning social isolation on spatial learning was discovered in the present study either, but the aforementioned results give rise to the question whether more differential reactions of heterozygous *Cacna1c*$^{+/-}$ rats and wild-type *Cacna1c*$^{+/+}$ controls might be observed in other maze paradigms.

Social and physical environmental enrichment, on the other hand, was found to have an overall positive effect on spatial and reversal learning. Rats previously exposed to environmental enrichment displayed the steepest reduction in time to criterion and the strongest decrease in error count, including reference and working memory errors. Cognitive flexibility was also highest in rats previously exposed to environmental enrichment. In fact, they showed patterns indicative of full cognitive flexibility on reversal learning Day 1. The positive effects of social and physical enrichment have previously been shown to enhance learning capabilities in the MWM (7,22,67), with enriched animals displaying shorter latencies and more direct paths to the target platform, faster improvement in general and more precise search strategies (30). Furthermore, environmental enrichment was found to improve habituation learning (11) as well as reference memory in a water-based radial maze in rats (48) and mice (7). Augmented reversal learning capabilities were also found in enriched mice tested in the MWM (22,30).

Gene × environment interactions in object recognition, spatial memory and reversal learning capabilities

Most importantly, however, we obtained evidence for G×E interactions in the present study, with environmental factors, in the form of post-weaning social isolation or social and physical enrichment, interacting with *Cacna1c* haploinsufficiency and exerting long-term effects on learning and memory. G×E interaction effects were most prominent during the reversal phase, as best reflected in time to criterion and error count, particularly reference memory errors. While in rats previously housed under standard conditions *Cacna1c*$^{+/-}$ rats performed worse than *Cacna1c*$^{+/+}$ controls at the level of time to criterion and error count, environmental enrichment with its overall positive effect on both genotypes caused them to perform equally well. This indicates that the exposure to positive environmental circumstances during critical time windows can mitigate the risk and detrimental course in *Cacna1c*-haploinsufficient rats.

Our findings are in line with recent evidence for G×E interactions in *Cacna1c* mouse models. Interestingly, when exposed to chronic unpredictable stress in a G×E interaction study, initial deficits in heterozygous *Cacna1c* mice turned into resilience later in life in a spontaneous alteration working memory task, as observed in a forebrain-specific heterozygous mouse model (6). Moreover, findings by Dedic *et al.* (18) suggest that G×E interactions are depending on the time-point during development, with manipulations early during development being particularly effective. Depletion of *Cacna1c* from forebrain glutamatergic neurons during early development led to increased susceptibility to chronic social defeat stress, while enhanced stress resilience was evident in adulthood. In their study, however, resilience was not evident at the level of cognitive functioning, with mice exposed to stress displaying impaired memory in the spatial object recognition test irrespective of genotype.

Reward sensitivity

Cacna1c-haploinsufficient rats did not show striking impairments in reward sensitivity in either genotype or experimental housing condition, similar to the rats tested before in our lab (10). However, Koppe *et al.* (43) hypothesized that while *Cacna1c* knockout animals were found not to be impaired in reward-based learning, they appear to employ a different strategy when searching for rewards. This is a matter that may be worth investigating in future experiments. Regarding environmental influence on reward sensitivity, rats previously exposed to social and physical environmental enrichment appeared to be the least sensitive to reward magnitude. This mirrors the general consensus that enriched animals exhibit poorer reward discrimination (40,80,86).

General Discussion

While we obtained evidence for G×E interactions in the present study, the long-term impact of environmental factors, in the form of post-weaning social isolation or social and physical enrichment, interacting with *Cacna1c* haploinsufficiency was moderate. First of all, it is important to emphasize that the positive effects of environmental enrichment were evident in adult rats housed in groups under standard conditions several

weeks after the exposure to social and physical environmental enrichment. Because all animals were housed with their littermates from other environmental conditions in the meantime, inclusion effects that have previously been found to normalize the influence of both genotype (87) and environment (69), cannot be precluded from affecting the results. Future studies might benefit from including same-genotype and same-environment housing following environmental manipulation. Stronger effects might also be expected when applying a shorter delay between the end of the environmental manipulation and the start of behavioral testing. Variations in task difficulty might as well help reveal more prominent G×E interactions, as hinted at by several studies that observed a distinct effect of enrichment depending on the inter-trial interval in the novel object recognition paradigm (53) or additional distracting stimuli (35). In fact, stronger *Cacna1c* genotype effects were also found under more difficult conditions, such as a limited cues version of the MWM (75) or longer intervals until probe trial testing (83).

Conclusion

In summary, our study provides evidence for a G×E interaction, i.e. an interplay between *Cacna1c* haploinsufficiency and environment during juvenile development, on object recognition, spatial memory and reversal learning capabilities. Social and physical enrichment had a positive influence on *Cacna1c*$^{+/-}$ rats and *Cacna1c*$^{+/+}$ littermate controls on spatial and reversal learning, while post-weaning social isolation negatively affected novel object recognition in both genotypes. Despite intact spatial learning and re-learning abilities in all groups, slight but consistent deficits were evident in *Cacna1c*$^{+/-}$ rats previously housed under standard conditions particularly during reversal learning but not *Cacna1c*$^{+/-}$ rats previously exposed to social and physical enrichment. Together, this supports the notion that *Cacna1c* interacts with the environment to shape disease vulnerability and associated alterations in cognitive functioning.

Materials and Methods

Animals

Constitutive 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 (24). *Cacna1c*$^{+/-}$ rats carry a 4 bp deletion at 460649–460652 bp in genomic sequence resulting in an early stop codon in exon 6. Previously, we have shown that $Ca_v1.2$ protein levels in the brain of *Cacna1c*$^{+/-}$ rats are reduced by ∼50% (41,42). Homozygous *Cacna1c*$^{-/-}$ rats were not used since they are embryonically lethal.

As reported before (41,42), 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 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. Rats were identified by paw tattoo, using non-toxic animal tattoo ink (Ketchum permanent tattoo inks green paste, Ketchum Manufacturing Inc., Brockville, Canada), inserted subcutaneously through a 30-gauge hypodermic needle tip into the center of the paw on PND 5 ± 1. Rats were housed under standard laboratory conditions (22 ± 2°C and 40–70% humidity) with free access to food and water, with the exceptions described below.

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 DNeasy 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).

Experimental housing conditions

To study the effects of a gene × environment (G×E) interaction effect on object recognition, spatial memory and reversal learning capabilities in rats, we applied a 2 × 3 experimental design and exposed male *Cacna1c*$^{+/-}$ rats and *Cacna1c*$^{+/+}$ littermate controls to one of three experimental housing conditions for 4 consecutive weeks after weaning on PND 21, i.e. between PND 22 and 50 ± 1 (Fig. 1). From each litter, $N = 6$ rats were included in the experiment whenever possible, with a pair of *Cacna1c*$^{+/-}$ and *Cacna1c*$^{+/+}$ siblings each being randomly exposed to one of three experimental housing conditions: (A) post-weaning social isolation (ISO), (B) standard housing (STD) or (C) social and physical environmental enrichment (ENR).

(A) ISO: post-weaning social isolation in a Makrolon type III cage (42.5 × 15.0 × 26.5 cm, length × width × height, plus high stainless-steel covers; Tecniplast Deutschland GmbH; Hohenpeißenberg, Germany), housed alone, applying a previously established protocol (69).
(B) STD: standard housing in a polycarbonate Makrolon Type IV cage (58 × 38 × 20 cm, plus high stainless-steel covers; Tecniplast Deutschland GmbH), housed in groups of $N = 6$ rats, consistent with previously applied control conditions (69).
(C) ENR: social and physical enrichment in a large rat cage (104 × 59 × 107 cm; AniOne Remus; MultiFit Tiernahrungs GmbH, Krefeld, Germany), housed in groups of $N = 6$, containing three wooden platforms connected by ramps, and an assortment of cage accessories and places to hide, applying a modified protocol previously established (11). The initial cage setup comprised six hiding places (2× Rodent Retreats red, Bio-Serv, Flemington, New Jersey, USA; 2× AniOne Grasnest Size L, MultiFit Tiernahrungs GmbH; 1× AniOne Grastunnel, MultiFit Tiernahrungs GmbH; 1× empty cardboard box, Dallmayr capsa, Munich, Germany), six wooden sticks for the animals to chew on and two petri dishes with water in addition to the water bottle affixed to the cage wall. Twice a week, various accessories were either added to the cage

(e.g. two wire balls stuffed with paper tissue, Food-Ball Ø 12 cm, TRIXIE Heimtierbedarf GmbH, Tarp, Germany; three marbles; two empty toilet paper rolls) or exchanged, in the case of hiding places (1× wooden rat house, Jesper Eckhaus Size M, TRIXIE Heimtierbedarf GmbH; 1× JR Farm Heuhaus 85 g, JR FARM GmbH, Holzheim-Pessenburgheim, Germany). During environmental manipulation, each enrichment group received two servings of six pieces of cereal (Kellogg's Smacks, Kellogg Deutschland, Hamburg, Germany), which were hidden within the cage.

In STD and ENR, two siblings were always housed with two further pairs of *Cacna1c*$^{+/-}$ and *Cacna1c*$^{+/+}$ siblings from different litters. To avoid age differences between litters, all rats included in the experiment were born within a 4-day time window. Following the 4-week exposure to different housing conditions, all rats were again socially housed in groups of 4–6 same-sex littermates in polycarbonate Makrolon Type IV cages under standard laboratory conditions, as described previously. In total, $N = 72$ rats were included in the experiment, with $N = 12$ per genotype and experimental housing condition (ISO$^{+/+}$, STD$^{+/+}$, ENR$^{+/+}$, ENR$^{+/-}$, STD$^{+/+}$, STD$^{+/-}$).

Behavioral phenotyping

As part of our longitudinal and comprehensive deep behavioral phenotyping approach (39), object recognition, spatial memory and reversal learning capabilities were assessed in male constitutive heterozygous *Cacna1c*$^{+/-}$ rats and examined in contrast to *Cacna1c*$^{+/+}$ littermate controls (Fig. 1). Behavior was compared within and between the three experimental housing conditions. Novel object recognition was assessed in male *Cacna1c*$^{+/-}$ rats and *Cacna1c*$^{+/+}$ littermate controls on PND 90–93. Spatial learning and re-learning was performed around PND 120–130. Before entering these paradigms, all animals were tested in other behavioral assays, namely playback of 50-kHz ultrasonic vocalizations, repetitive behavior, open field and elevated plus-maze. Novel object recognition was tested in $N = 36$ male *Cacna1c*$^{+/-}$ rats and $N = 36$ male *Cacna1c*$^{+/+}$ littermates. Spatial and reversal learning was assessed in $N = 18$ male *Cacna1c*$^{+/-}$ rats and $N = 18$ male *Cacna1c*$^{+/+}$ littermates. In both paradigms, a third of each group had previously undergone one of the three housing conditions (ISO, STD, ENR), as described previously. 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. Prior to the first behavioral paradigm, all animals were handled (PND 56 ± 2) using a standard handling protocol.

Novel object recognition

Around PND 90–93, the novel object recognition test was conducted in an open field, as described previously (10). 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. 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 into open field, where the two identical objects had been located during 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.

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 lx) 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 (25) and as described in Braun *et al.* (10). Spatial and reversal learning was performed on a radial eight-arm maze. The arms extended radially from a central platform and were numbered in a clockwise fashion from 1 to 8. Four centimeters from the distal end of each arm, a food pit 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, 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. Food deprivation started 7 days before the beginning of the spatial and reversal learning task to familiarize the rats with the restricted food regimen. To avoid aggressive behavior sometimes occurring during food deprivation, rats were individually housed. 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 7 days, immediately followed by the reversal learning period, which also lasted 7 days. Initially, the rats were exposed to the food pellets later used as reward (45 mg, Bio-Serv 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 cutoff 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 7 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 cutoff 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 from about 150 cm above the radial maze. For behavioral analysis, an experienced observer scored the videos for the type of arm entries 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 were tested under dim white light (70 lx) conditions.

Statistical analysis

All statistical tests were carried out using IBM SPSS Statistics (Version 24.0) software. 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 (1–7) and between-subject factors genotype (*Cacna1c*$^{+/+}$ vs. *Cacna1c*$^{+/-}$) and experimental environment (ISO vs. STD vs. ENR). Error counts were always averaged for each day of learning and converted into percentages of made entries. For the comparison of Days 1 and 7 of learning within each group, *t*-tests for paired samples were conducted. Arm preference during the probe trials was analyzed using paired one-tailed *t*-tests, comparing entries into baited and entries into non-baited arms for each of the six experimental groups. Differences in preference between genotypes or environments were assessed by an ANOVA for each arm type with between-subject factors genotype and experimental environment. 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 within each group. 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 and housing differences (environment) in general exploration of all objects were analyzed with one ANOVA per trial and between-subject factors genotype and environment. A P value of < 0.050 was considered statistically significant (D = main effect of training day; G = main effect of genotype; E = main effect of experimental housing condition; × = interaction effect, e.g. D×G = interaction of training day and genotype). All values were reported as mean ± standard error means (SEM). For all post hoc analyses, Tukey HSD tests were employed and considered significant at a P value of < 0.050.

Supplementary Material

Supplementary Material is available at *HMG* online.

Author contributions

M.W. conceived the study; M.B. and T.K. performed the experiments; M.B. analyzed the data; M.B. and M.W. wrote the manuscript with the help of all other authors; S.W., M.R., R.S. and M.W. acquired funding.

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General discussion

Several risk variants of *CACNA1C* were found to be overrepresented in populations with neuropsychiatric disorders such as MDD, BPD, SCZ and ASD. These disorders are characterized by severe and debilitating deficits in social interaction and communication, as well as widespread cognitive dysfunction which transcends diagnostic boundaries and imposes a great burden on the affected individuals and their caregivers. Various findings from both human and mouse studies have suggested that *CACNA1C* plays a role in the etiology of these observed social, learning and memory impairments, yet the exact mechanisms and their interplay with environmental influences remain obscure. Both *Cacna1c* knock-in and knockout models have previously been generated in mice, and both approaches have discovered social impairments (Bader et al., 2011; Dedic et al., 2018; Jeon et al., 2010; Kabir et al., 2017a) as well as diminished learning capabilities (Bader et al., 2011; Dedic et al., 2018; Moosmang et al., 2005b; Temme et al., 2016). Since the scientific community still lacks a deep understanding of the exact relationship between *CACNA1C* risk variants, $Ca_v1.2$ channel function and associated behavioral alterations, this thesis set out in an attempt to elucidate how the *CACNA1C* gene confers risk to these intermediate phenotypes and interacts with positive or negative environmental factors to ameliorate or exacerbate certain behaviors using a transgenic rodent model. To this aim, we examined a newly generated *Cacna1c* constitutive HET knockout rat model in various paradigms with validity to human social and cognitive behaviors.

The results from Studies I and II suggest that a global *Cacna1c* haploinsufficiency induces prominent sex-specific alterations in social play behavior and communication deficits during adolescence in both the sender and receiver of 50-kHz calls. By comparison, Study III and IV discovered less striking impairments of cognitive function due to differences in genotype. *Cacna1c* heterozygosity was associated with superior spatial learning but reduced cognitive flexibility during reversal learning in a sex-dependent manner. Regarding housing conditions, ENR during a critical post-weaning developmental period enhanced spatial and reversal learning in adulthood, as well as cognitive flexibility readouts on reversal learning day 1. Social isolation slightly affected spatial learning in a negative fashion and caused a strong impairment in object recognition abilities. Further, a GxE interaction was indicated by the positive effects of ENR on the SL and RL performance of HET *Cacna1c* rats, helping them to be on par with WT littermates instead of delivering an inferior performance under STD housing.

In the following sections, I will evaluate these results in the context of previous findings and regarding their implications for human behavior. Using the reward circuit and the frontolimbic system as examples, I will go into detail regarding potential biological mechanisms and associated neuronal

structures by which *CACNA1C* and manipulations like ISO and ENR may influence social behavior, spatial learning and relearning, as well as novel object recognition. Beginning with genotype effects on social behavior and cognitive abilities, I will move on to the influence of environmental manipulation on learning and memory measures and then focus on our GxE findings. Subsequently, I will discuss the methodological specifics that need to be considered in the discussion of the experiments in this dissertation and conclude with an evaluation of the studies' limitations and potential applications for clinical research and refinements in future experiments.

Influence of *Cacna1c* haploinsufficiency on social behavior

Social deficits like diminished interest in social contact and social withdrawal, impaired communication and reduced empathy are common to all major neuropsychiatric disorders (Ferretti & Papaleo, 2019; Porcelli et al., 2019; Sanchez-Moreno et al., 2009). Unfortunately, the exact etiology of social deficits and their role in disease manifestation remain to be elucidated, but various components of altered social behavior, like low extraversion (Roussos et al., 2011), greater hostility (Takeuchi et al., 2018) and deviant facial affect processing (Dima et al., 2013; Nieratschker et al., 2015; Soeiro-de-Souza et al., 2012) have already been found to be associated with *CACNA1C* risk variants in clinical studies. As rats are highly gregarious creatures that can be readily employed in a wide range of assays investigating social behavior, we used our newly generated constitutive *Cacna1c* HET knockout model to investigate the effects of the gene on social interaction and communication in rats on a Sprague-Dawley background. For this, we utilized the natural tendency of juvenile rats to engage in rough-and-tumble play (Panksepp, 1981), in combination with the assessment of USV production during playful interactions (Lukas & Wöhr, 2015), as well as the established social approach reaction to playback of positively valanced 50-kHz calls (Wöhr & Schwarting, 2007).

Play behavior & social interaction

Previous findings in *Cacna1c* mice have yielded inconsistent results regarding social interaction (Bader et al., 2011; Dedic et al., 2018; Kabitzke et al., 2018). As highlighted before, the social behavioral repertoire of rats is considered to be significantly broader than that of mice, especially concerning their use of USV and the display of rough-and-tumble play (Berg et al., 2018; Ellenbroek & Youn, 2016), which is why we expected to find prominent deficits in the social behavior of juvenile rats with constitutive HET *Cacna1c* deletion. While we did observe distinct social deficits in some respects, the relationship with *Cacna1c* haploinsufficiency appears to be sex-dependent. Play behavior was unaltered in male rats, which is contrary to what we hypothesized and indicates that play behavior itself is not affected by *Cacna1c* expression in males. Contrarily, we observed a significant increase in playful interactions in female HET dyads compared to WT controls. In fact, the duration and frequency

of play in HET females across the three days exceeded even that of WT Sprague-Dawley males, which are usually known to engage in rough-and-tumble play to a greater extent than their female counterparts (Argue & McCarthy, 2015; Beatty et al., 1981). Pinning behavior, specifically, was markedly increased in *Cacna1c* haploinsufficient females. This behavior is characterized by one play partner lying on their back in a defensive position with the other animal standing on top, and is also more commonly observed in male-male interactions than in female pairs (Argue & McCarthy, 2015). In behaviors outside of those specific to play behavior, such as general sniffing and physical contact, no genotype differences were found. Together, these results indicate that the deletion of *Cacna1c* on one allele results in significantly increased social interaction and a more masculine play style with elevated pinning in female rats.

Behavior	**Paradigm**	**male HETs**	**female HETs**
Social interaction	Rough-and-tumble play	NE	↑ play-specific interactions ↑ pinning moves
USV emission	Rough-and-tumble play	↓ number of calls ↓ peak amplitude ↑ peak frequency	NE
USV reception	Playback of 50kHz USV	↓ social approach ↓ sustained interest ↓ white noise avoidance	↓ sustained interest

Table 5: Summary of key findings: *Cacna1c* effects on social behavior in Study I and II. Effect direction always refers to the comparison with wildtype littermate controls of the same sex. ↓ = decreased. ↑ = increased. NE = no effect. HET = heterozygous. USV = ultrasonic vocalization(s).

Our results are partly in line with available research on *Cacna1c* mice. Two studies inactivated *Cacna1c* in forebrain glutamatergic neurons during development and found a decreased preference for the social stimulus in male mice subjected to the three-chamber test (Dedic et al., 2018; Kabir et al., 2017a). Furthermore, region-specific effects were indicated by a similarly reduced sociability in mice with a focal knockdown of *Cacna1c* in the adult PFC (Kabir et al., 2017a), but not in the NAcc (Terrillion et al., 2017b). Then again, constitutive HET mice, the mouse pendant to our rats, showed unaltered social approach behavior in the three-chamber test (Dedic et al., 2018), and a sustained preference for the social stimulus in a newly developed assay within home-cage environment (Bader et al., 2011). In the same paradigm, TS2-neo mice were found to exhibit a mild reduction in sociability, as well as stronger impairments in a prolonged version of the three-chamber test (Bader et al., 2011). It has to be said, though, that in contrast to our genetic manipulation, this latter study employed a gain-of-function model where the inactivation of $Ca_v1.2$ is considerably reduced (Splawski et al., 2004). Regrettably, research on social interaction in *Cacna1c* rats, female animals, as well as juveniles is sparse. However, in a recent publication from our lab using the same genetic model, mild social impairments during direct reciprocal social interaction were observed in adult female *Cacna1c* HET

rats (Redecker et al., 2019), when allowed to interact with a same-sex conspecific for 10 minutes. Females with partial *Cacna1c* ablation were found to be more socially dominant than WT controls without showing aggressive behavior, which agrees with our findings of increased pinning in HET females. Of note, animals were allowed to interact with partners of both genotypes in this study and HET rats seemingly adjusted their behavior according to the genotype of their partner, whereas WT animals did not. Specifically, more social interactions were observed if HETs were paired with other HETs than in mixed dyads, indicating a preference for same-genotype interactions (Redecker et al., 2019).

In combination with these studies on mice and rats, our evidence suggests that alterations in *Cacna1c* expression, especially in the PFC (Kabir et al., 2017a), lead to aberrant social interaction in a variety of paradigms.

USV during play & social cue emission

Next to general play behavior, we assessed USV during rough-and-tumble play. Increased play behavior was not concomitant with increased 50-kHz USV emission in HET females, who exhibited a similar rate of USV production as their WT counterparts during play. Inverted result patterns were obtained in HET males whose play behavior was comparable to animals with full *Cacna1c* expression yet accompanied by an altered emission of prosocial 50-kHz USV. In contrast to WT siblings, they produced 50-kHz calls at significantly lower levels and at higher peak frequencies, yet lower peak amplitudes during play. Regarding call subtypes, a strong reduction of FLAT and MIXED calls was identified, which have previously been associated with distinct affective states and communicative functions (Burke et al., 2017c; Himmler et al., 2014), like the re-establishment of social proximity and the synchronization of complex social interactions (Łopuch & Popik, 2011). The decreased call production in male HETs in Study I can be interpreted as a sign of diminished positive affective state during play (Burgdorf et al., 2011). Alternatively, the relationship between 50-kHz USV and positive valence may not be as robust in haploinsufficient males as it is in WT animals. This latter interpretation, however, appears incongruent to the increased call production seen in advance of play sessions, which is often used as a behavioral readout for the motivation to play (Knutson et al., 1998). There were no genotype differences in 50-kHz calling in anticipation of rough-and-tumble play, indicating that while male HET animals were motivated to play, they did not derive the same reward from the interaction as their WT siblings.

Regarding the investigation of deficits in the social domain linked to *Cacna1c*, most studies on mice have so far focused on social interaction and social recognition, but social communication and motivation have not been studied to a great extent (Young et al., 2009). Up until this point, the only

communicative findings in mice have been obtained in the TS2-neo model. In one study, pup USV of genetically manipulated animals turned out to be significantly shorter than in controls during the first two weeks of life (Bader et al., 2011), yet in another, no genotype difference in USV production was discovered (Kabitzke et al., 2018). It must be noted, though, that by comparison, the relevance of USV for socio-affective communication in rats may be decidedly higher (Ferretti & Papaleo, 2019). In rats, *Cacna1c* appears to not only influence USV production during the juvenile phase but also during adulthood, as highlighted by a recent study from our lab using the same genetic model (Redecker et al., 2019). Data from this line of experiments shows that during direct reciprocal interaction of adult female rats, a strongly reduced 50-kHz USV emission was displayed by haploinsufficient animals on both test days, and in social, as well as non-social periods of the interaction, which is consistent with findings from Study I. A replication of our lower peak amplitude finding in these animals suggest that this alteration is stable across development. Notably, and like in Study I, the deficits in social communication displayed by the adult female HETs in this experiment did not lead to reduced social contact, but rather to an enhanced duration of physical proximity in dyads including a HET female (Redecker et al., 2019). 50-kHz USV in general were primarily emitted during social periods, however, underscoring the role of USV in rodent communication. Indeed, evidence for the communicative function in social play, specifically, has been provided by several studies. Rats selectively bred for high emission of 50-kHz calls have been found to engage more in rough-and-tumble play (Panksepp & Burgdorf, 2000), whereas when prohibited from producing 50-kHz USV, they develop severe social impairments. As a matter of fact, social play behavior in particular is impaired in deaf, as well as devocalized animals (Kisko et al., 2015b; Siviy & Panksepp, 1987). Of particular relevance to the findings from Study II in this context is the discovery that in rats selectively bred for low levels of 50-kHz calling, *Cacna1c* gene expression was found to be altered (Moskal et al., 2011) and concomitant with increased pinning behavior (Webber et al., 2012), further implicating the gene in aberrant social communication.

USV playback & social cue reception

A large body of evidence suggests that the playback of 50-kHz calls usually elicits exploration and social approach (Seffer et al., 2014; Willadsen et al., 2014; Wöhr & Schwarting, 2009, 2012) which is often used as a behavioral measure for social motivation (Engelhardt et al., 2017), whereas the playback of 22-kHz calls is generally known to induce freezing and behavioral inhibition (Fendt et al., 2018; Wöhr & Schwarting, 2007).

In Study I and II, we exposed *Cacna1c* HET rats and WT siblings to 50-kHz USV on a radial arm maze. Both male and female HETs demonstrated a significantly altered reaction to social cues. Even though

prominent social approach behavior is commonly observed in male rats, specifically, HET males showed noticeably reduced social approach to 50-kHz USV playback compared to WT littermates and did not keep searching for the sound source in the minutes following the stimulus. Furthermore, male HETs did not display avoidance behavior at white noise playback, whereas WT rats did. HET females displayed similar patterns of behavior, albeit in a more moderate form. Females of both genotypes approached the active speaker at the presentation of 50-kHz calls and demonstrated a preference for proximal arms near to the sound source, but while WT females showed sustained exploration of the proximal arms in an effort to locate the sender of 50-kHz calls, HET females quickly lost interest in the minutes following stimulus presentation. This is in accordance with our findings in HET males in Study I, as well as with a paper on the sociability in TS2-neo mice. Whereas these animals showed intact social preference during the regular three chamber-test, the genetically manipulated mice lost interest significantly sooner than their WT counterparts in a prolonged version of the paradigm (Bader et al., 2011), indicating that *Cacna1c* is involved in the regulation of sustained motivation for social contact.

Judging by the number of publications, little research is conducted on phenotypes with relevance to social cue reception in the context of *Cacna1c*, even though the inability to process social signals can lead to severe social dysfunction (Meyer-Lindenberg & Tost, 2012). Interesting evidence was obtained, however, in a study using mice harboring a local $Ca_V1.2$ knockout in the ACC in an observational fear learning paradigm (Jeon et al., 2010). In this experiment, which is aimed at the acquisition of a fear conditioned stimulus in one mouse that merely observes another one receiving a foot shock, but is not exposed to the noxious stimulus itself, knockout animals displayed markedly reduced levels of vicarious freezing. Notably, regular fear conditioning was unimpaired in ACC knockout mice, yet the social component of observational fear conditioning, in particular, was affected. This indicates that *Cacna1c* expression in the ACC may be involved in the process of recognizing affective cues in conspecifics and inferring to their mental state (Jeon et al., 2010). No other experiment has so far employed 50-kHz USV playback paradigms, yet there is evidence from a recent study in our group, implicating *Cacna1c* expression levels in the response to 22-kHz USV playback, too (Wöhr et al., 2019). Of note, no behavioral alterations to 22-kHz calls were observed in *Cacna1c* females. However, male rats haploinsufficient for the *Cacna1c* gene were found to be significantly less inhibited by 22-kHz USV playback than WT controls, and to display similar reactions to natural calls and phase-scrambled control noises (Wöhr et al., 2019), altogether indicating that production, as well as processing of social signals is altered in constitutive *Cacna1c* heterozygous rats.

As mentioned previously, rats are also known to respond to 50-kHz USV playback with 22-kHz calls if they are unable to locate the source of these social contact calls (Berg et al., 2018; Coffey et al., 2013). This was also true for the animals examined in Study II. Interestingly, the number of 22-kHz frustration

calls was lowest in HET females, suggesting a diminished level of frustration (Wöhr & Schwarting, 2009) and potentially reduced interest in the search for conspecifics.

Sex differences in social behavior

Regarding social communication, *Cacna1c* males appear to be more strongly affected by *Cacna1c* haploinsufficiency than females, especially considering the low levels of 50-kHz USV emission during play, the more prominently reduced approach behavior elicited by pro-social 50-kHz calls in Study I, as well as the attenuated inhibition following 22-kHz USV exposure (Wöhr et al., 2019). Then again, the overt behavior during social interactions of female HETs appears to be more vulnerable to partial *Cacna1c* depletion than that of male HETs, demonstrated in Study II by their extraordinarily high level of rough-and-tumble play, especially regarding pinning, as well as their more masculine style of playing in general. Interestingly, new evidence from our lab indicates that while at first glance, USV production during play is unaltered in the female HETs compared to wildtype littermates in Study II, a detailed analysis of the acoustic parameters can reveal a more male-like call subtype profile (Kisko et al., 2020). These observations concur with findings of increasingly dominant behavior of HETs towards WT females and reduced 50-kHz vocalizations in direct reciprocal social interactions (Redecker et al., 2019), and are further corroborated by indications that in humans, female *CACNA1C* risk allele carriers exhibit tendencies towards greater hostility and reduced interpersonal emotional intelligence (Takeuchi et al., 2018).

Social dysfunction in various neuropsychiatric disorders has been suspected to affect males more strongly than females (Vaskinn et al., 2011), and at least for social communication, this holds true for *Cacna1c*-specific effects on social communication in the animals of Study I and II as well. Astonishingly, though, partial constitutive ablation of *Cacna1c* provoked our female HETs to engage in social play at levels that even surpassed those of WT males, which is especially surprising considering the fact that typically, male rats are known to play more than females (Pellis et al., 1994; Thor & Holloway, 1986). One mechanism by which *Cacna1c* possibly exerts its sex-dependent influence on social behavior is via hormonal changes. These are considered to be especially pertinent in the adolescent period our experiments were conducted in, and – in the case of ovarian hormones – have been shown to be relevant for the maintenance of female-like patterns of play behavior (Burke et al., 2017a). Injections of testosterone, on the other hand, have been revealed to defeminize female play behavior (Pellis et al., 1994). Interestingly, several brain regions like the amygdala, which is strongly implicated in social behavior (Porcelli et al., 2019), are not only differentially affected by sex hormones (Meaney & McEwen, 1986), but also by *CACNA1C* (Jogia et al., 2011; Perrier et al., 2011; Tesli et al., 2013; Wessa et al., 2010).

Alas, while several publications have investigated *Cacna1c*-related sex differences in mice (Dao et al., 2010; Lee et al., 2012; Zanos et al., 2015), there is a prominent lack of studies contrasting the effects of *Cacna1c* on social interaction in male and female animals which makes it difficult to provide definite answers as to whether the differences of our social interaction findings compared to previous studies in mice are due to sex-, task-, age-, species- or brain region-associated factors. Nevertheless, the results from Study I and II provide strong evidence that a partial constitutive depletion of *Cacna1c* leads to a significantly diminished ability to produce and to adequately respond to socio-affective communication signals in rats. *Cacna1c* haploinsufficiency furthermore appears to exert a sex-dependent influence on social interaction, such as rough-and-tumble play.

Implications for social communication deficits

As noted above, most research has so far focused on social preference and direct interactions. Clinical studies, however, indicate that adequate behavior in social situations is strongly based on the ability to understand and participate in social communication, and may be encumbered either due to deficits in social stimulus interpretation, or in cue perception and salience coding. In humans, for example, diminished attention to social cues has been shown to increase the risk for emotional dysregulation and aggressive behavior in abused children (Dodge et al., 1990). Intriguingly, impairments in social stimulus processing are common symptoms of several mental disorders and have already been associated with *CACNA1C* risk variants on a behavioral level. SCZ and ASD, for instance, usually manifest in different periods of life, but both are linked to deficits in social information processing (Eack et al., 2013; Pu et al., 2019) and especially in the latter, nonverbal and verbal communication deficits, such as delayed language acquisition and abnormal prosody or poor eye contact are common phenomena in autistic children (DiLalla et al., 2017; Millan et al., 2012). Indeed, impairments in emotion recognition have been described for all four of the disorders covered so far in this thesis (Nieratschker et al., 2015). Regarding genetic influence, deficits in facial affect recognition have already been connected to the rs1006737 risk allele in patients of bipolar disorder (Soeiro-de-Souza et al., 2012) as well as healthy subjects (Nieratschker et al., 2015). In the rat model employed in Study I and II, altered responses to pro-social 50-kHz calls, 22-kHz alarm calls, as well as to non-social stimuli were found in animals with constitutive *Cacna1c* haploinsufficiency. Together, this indicates that *Cacna1c* expression may somehow modify the relevance or salience of social cues in these animals.

In terms of brain correlates, the structure most associated with this task – and therefore of interest for this discussion – is the amygdala (Porcelli et al., 2019). It is widely accepted as a fact that the amygdala is involved in a plethora of social functions in rodents, humans and non-human primates (Felix-Ortiz & Tye, 2014). In rodents, the amygdala has been shown to mediate social behavior (Kabir et al., 2016),

like the emission of 22-kHz USV during e.g. aversive situations (Wöhr & Scattoni, 2013) and social olfactory processing (Ferretti & Papaleo, 2019), and lesions in the amygdala evidently influence the specifics of play behavior in rats (Meaney et al., 1981). In humans, social perception, affiliation and social aversion processes are all thought to touch the amygdala at least once on the way to a behavioral outcome, and the structure is indeed believed to be a central hub for the orchestration of social behavior (Porcelli et al., 2019). The control of neural activity in the amygdala appears to be quite complex, though, seeing that increased activity can be measured during social interaction behaviors (Felix-Ortiz & Tye, 2014), yet hyperactivity is associated with social avoidance (Drevets et al., 2008; Porcelli et al., 2019). Of particular importance for the findings from Study I and II is the notion that the amygdala modulates the attention to external stimuli (Ferretti & Papaleo, 2019) and encodes their affective valence (Morris et al., 1996). Indeed, the low desire for social contact in disorders like MDD and SCZ has been hypothesized to be owed to the missing attribution of positive relevance to social stimuli (Kahn et al., 2015; Kupferberg et al., 2016), and *CACNA1C* risk variants have been strongly implicated in the anatomical and functional deviations in the amygdala discovered in various studies on patients of MDD (Hasler et al., 2004; Koch et al., 2019), BPD (Drevets et al., 2008; Tesli et al., 2013), SCZ (Kahn et al., 2015; Powell et al., 2015) and ASD (Grady & Keightley, 2002; Lai et al., 2014). Structural findings include enlarged amygdala volume (Lancaster et al., 2016; Perrier et al., 2011) as well as microstructural alterations (Koch et al., 2019) in SNP rs1006737 risk allele carriers. One of the most consistent discoveries in clinical studies of *CACNA1C*, however, is the association of risk variants with amygdala dysfunction in BPD patients and healthy controls. In several functional MRI studies examining facial affect processing, like the negative face matching task or fear-face recognition, these subjects consistently showed higher amygdala activity than non-risk allele carriers (Dima et al., 2013; Jogia et al., 2011; Tesli et al., 2013). However, these aberrations in social cue processing are not restricted to mere negatively valanced stimuli. In fact, in one study, healthy participants homozygous for the *CACNA1C* risk variant exhibited strongly altered salience processing, as they specifically rated pleasant and unpleasant stimuli alike as less arousing than any neutral pictures presented to them (Pasparakis et al., 2015).

In view of these discoveries, it seems conceivable that risk variants in *CACNA1C* alter the anatomy of and activity within the amygdala to influence physiological responses during salience processing, as well as subsequent behavioral reactions to affective stimuli like facial expressions in humans or pro-social 50-kHz USV in the rats of Study I and II.

Implications for social motivation deficits

Our results also indicate that altered *Cacna1c* expression levels may be involved in the desire for and pleasure derived from social interaction. As previously noted, social withdrawal is one of the earliest signs of social dysfunction in many disorders (Young et al., 2009), often concomitant with a significant reduction of interest in ("wanting") and enjoyment of ("liking") social affiliation (Berridge et al., 2009), commonly referred to as social anhedonia. In MDD, this phenomenon is correlated with the severity of depressive symptoms and can predict non-response to antidepressants, as well as psychotherapy (Kupferberg et al., 2016).

In rats, play behavior during juvenility is typically considered to be highly rewarding to the participants (Trezza et al., 2010; Vanderschuren et al., 2016), and as USV emission has been hypothesized to reflect the affective state in the sender (Burgdorf & Panksepp, 2001; Knutson et al., 1999; Panksepp, 2005), the marked reduction in the vocalization levels of HET males during play observed consistently in Study I and II suggests that these animals derive less pleasure from the interaction and thus experience less "liking" of social contact (Berridge et al., 2009). In females, USV production during play was not affected by *Cacna1c* genotype, implying at first glance that HET females derive the same level of reward from play as their WT siblings. However, when examining the details in their behavior, the significant increase of pinning moves in HET females paints a slightly different picture. Interestingly, pinning is considered to be one of the most rewarding aspects of rough-and-tumble play (Panksepp, 1981), and indeed, the animal actively pinning down their play partner is assumed to derive the greater reward from this move (Kisko et al., 2015a). Considering the comparable levels of 50-kHz vocalizations between genotypes, this suggests that HET females may need to engage in this type of playful interaction considerably more often to derive the same level of positive reinforcement from play as WT littermates. In this context, another study from our lab has recently shown that in adulthood, *Cacna1c* HET females also emit significantly fewer 50-kHz calls in direct reciprocal social interaction than WT controls (Redecker et al., 2019). Together, these findings point towards reduced "liking" of social interaction in both male and female rats haploinsufficient for the *Cacna1c* gene.

Additionally, active approach towards the playback of pro-social 50-kHz USV playback is often used as a behavioral readout for the incentive value of social stimuli in rats (Engelhardt et al., 2018). In Study I, HET males were found to exhibit profoundly diminished approach behavior towards pro-social 50-kHz USV playback, which is often interpreted as a sign of reduced "wanting" of social contact, i.e. the effort deemed acceptable to obtain a reward (Berridge et al., 2009). In the female rats of Study II, this alteration was not displayed during the 50-kHz call presentation itself, yet in haploinsufficient animals, sustained interest in the search for the sound source was impaired in the minutes following the

stimulus. In brief, *Cacna1c* HET rats of both sexes exhibit behaviors indicative of impaired "wanting" and "liking" of usually reward-associated social interaction, suggesting a role for the gene in processes that ensure social affiliation is experienced as rewarding. This impediment can have detrimental consequences on social function in general, as it is considered necessary to associate social contact with reward to develop and maintain strong social bonds which can ultimately act as protective factors buffering the negative consequences of stress and trauma (Alloy et al., 2005; Kendler et al., 2005; Porcelli et al., 2019). Persuasive evidence for this theory has been obtained in clinical studies that discovered a link between impaired reward function and the *CACNA1C* risk SNP rs1006737 (Lancaster et al., 2014), as well as in studies focusing on brain structures belonging to the reward circuit.

Regarding neurobiological correlates, responses to reward and general reward processing are most often associated with dopaminergic signaling in the mesolimbic pathway (Dichter et al., 2012) via the Nucleus accumbens (NAcc) and the ventral tegmental area (VTA), for the firing of neurons in these regions has been observed specifically during the prediction of reward, as well as when an expected reward is withheld (Miller, 2000). Dopaminergic cells in the VTA and NAcc are also thought to regulate motivation (Liu et al., 2014) and to code for the incentive values of natural rewards such as food or sexual intercourse (Dere et al., 2010). Interestingly, the mesolimbic pathway is also active in situations of particular relevance to social function, such as when viewing a picture of a loved one (Porcelli et al., 2019) or at the approach of close friends (Kupferberg et al., 2016). Alterations in reward circuitry have been related to the susceptibility to social defeat (Krishnan et al., 2007). In rats, dopaminergic signaling in the NAcc and VTA is strongly linked to the emission of 50-kHz calls (Burgdorf et al., 2001; Willuhn et al., 2014) and social play behavior (van Kerkhof et al., 2014; Vanderschuren et al., 2016).

Besides being altered in various mental disorders (Dichter et al., 2012; Grace, 2016; Whitton et al., 2015), these areas of neuroanatomy and associated dopaminergic signaling are also impacted by variations in *CACNA1C*. For example, not only have LTCCs been consistently linked to burst firing of dopaminergic neurons in the VTA (Liu et al., 2014) and behavioral sensitization in drug studies, but the inactivation of LTCCs can also reduce the reinforcing effects of psychostimulants like amphetamine (Berger & Bartsch, 2014). In terms of $Ca_v1.2$-specific studies, the volume of the NAcc has been found enlarged in human risk allele carriers (Frazier et al., 2014) and *Cacna1c* has additionally been associated with altered DA-signaling in relevant mouse models (Kabir et al., 2016). Specifically, the changes of molecular properties of the VTA and NAcc due to social stress (Krishnan et al., 2007) appear to be mediated by *Cacna1c*. In mice where *Cacna1c* was knocked down virally in the NAcc, subthreshold social defeat stress resulted in impaired social interactions, whereas in genetically unaltered mice, this had no negative impact on social behavior (Terrillion et al., 2017b). Furthermore, several intriguing lines of evidence suggest *Cacna1c* may be crucial for the behavioral response to dopamine stimulants.

For instance, it was ascertained that the administration of AMPH leads to an attenuated hyperlocomotor response in mice harboring a partial *Cacna1c* deletion (Dao et al., 2010; Terrillion et al., 2017a), and $Ca_v1.2$ mRNA and protein levels were increased in the VTA of rats exposed to chronic AMPH treatment compared to drug-naïve animals (Rajadhyaksha et al., 2004). Apparently, *Cacna1c* specifically influences reuptake of dopamine in the VTA, but is also thought to exert an effect on downstream projections to the NAcc (Terrillion et al., 2017a).

In light of these explanations, our results can be interpreted as convincing evidence of diminished incentive salience of 50-kHz USV in both *Cacna1c* haploinsufficient senders and receivers, potentially mediated by dopamine signaling in mesolimbic reward pathway. Together, Studies I and II add to the growing body of literature that suggests *Cacna1c* expression levels are highly relevant for behaviors associated with social anhedonia, social withdrawal, and communication deficits in various disorders. Notably, our rat model shows translational promise for several neuropsychiatric disorders linked to alterations in the social domain, but especially for ASD, as in a recent study from our lab, these animals also displayed tendencies towards repetitive behavior during self-grooming (Redecker et al., 2019).

Influence of *Cacna1c* haploinsufficiency on cognition

Cognitive dysfunction, such as impaired recall from long-term episodic memory, impaired recognition, but also deficits in terms of executive function and working memory are common to all major neuropsychiatric disorders (Millan et al., 2012). Already, risk variants in the *CACNA1C* gene have been linked to altered memory function on the behavioral level in clinical studies, with risk allele carriers demonstrating worse logical memory abilities (Hori et al., 2012), decreased verbal fluency (Backes et al., 2014; Krug et al., 2010) and generally poor performance in a variety of cognitive tests (Dietsche et al., 2014).

Next to mice, which most animal models for *Cacna1c* have so far focused on (Kabir et al., 2016; Moon et al., 2018), rats are commonly used in translational research (Homberg et al., 2017). As highly intelligent animals, they can be used in wide range of cognitive assays and are known to deliver a stable learning performance (Ellenbroek & Youn, 2016). Based on the findings in *Cacna1c* mouse studies, we expected to find prominent deficits in spatial learning, reversal learning and novel object recognition abilities in Studies III and IV. However, in contrast to our initial hypotheses, constitutive HET *Cacna1c* rats displayed only mild deficits in cognitive flexibility, whereas in some other cognitive measures, they even delivered an above normal performance.

Spatial learning

Cacna1c haploinsufficiency does not seem to impair spatial learning abilities in the RAM in general. Although WT rats do display a steeper learning curve in Study III, HET rats were quite able to acquire the rewarded arm configuration within seven days and developed particularly strong memory traces, as displayed during the following probe trial. In HET females, we even observed an increased efficiency in collecting the food rewards during the first few days of SL, as they displayed hypolocomotion at a comparable, if not better level of accuracy. This reduced locomotion did not persist through the spatial learning phase and did not impair their ability to perform well in the subsequent probe trial.

Construct	Paradigm	male HETs	female HETs
Spatial Learning	Radial arm maze SL	↓ rate of error reduction ↑ probe trial performance	↓ number of arm entries ↓ number of mixed errors
Working Memory	Radial arm maze SL+RL	NE	NE
Reversal Learning	Radial arm maze RL	↓ rate of error reduction ↓ flexibility on RL1 ↑ probe trial performance	NE
Reward sensitivity	Radial arm maze SL+RL	NE	NE
Object recognition	Novel object recognition	NE	NE

Table 6: Summary of key findings: *Cacna1c* effects on cognition in Study III and IV. Effect direction always refers to the comparison with wildtype littermate controls of the same sex. ↓ = decreased. ↑ = increased. NE = no effect. HET = heterozygous. SL = spatial learning. RL = reversal learning.

Previous studies investigating the effect of *Cacna1c* deletion in mice have observed somewhat conflicting results. In one of the very first translational *Cacna1c* papers, the authors tested male mice harboring a conditional knockout for *Cacna1c* in the hippocampus and neocortex in the Morris water maze (Moosmang et al., 2005a). Their results partially match those from Study III in that initially, no major learning differences were observed between knockout and control mice, except a slight, but significant superiority of control mice in terms of escape latencies. However, after the second day of training, the progress of knockout mice in making correct choices in the search for the escape platform stagnated, whereas WT animals improved their performance noticeably, suggesting a strong deficit of spatial learning abilities in conditional knockout mice. Similar results were obtained by White and colleagues, although in their study, the impairments manifested much later. While both knockout and control mice were able to successfully learn the MWM over 14 days, as displayed by more and more reduced escape latencies, a significantly worse performance was displayed in a remote probe trial by knockout animals, even though no deficits were observed in a probe trial 24h after training (White et al., 2008). Since the remote probe trial was conducted 30 days after training, this behavior is indicative of less robust long-term memory traces in knockout mice. By contrast, the HET rats surveyed in Study III showed particularly strong memory traces directly after testing on spatial learning day 7. We did not

test memory retention in a remote probe trial, but it is conceivable that a time-dependent nature of cognitive deficits causes them to not be immediately apparent.

Then again, in a TS2-neo model, mice were trained in the MWM for five consecutive days and showed normal acquisition of the platform location, as well as intact spatial memories in a probe trial administered one day after training completion, consequently raising the question if instead of timing, the specificity of the genetic knockout might influence cognitive behavioral outcomes (Bader et al., 2011). This latter notion is supported by findings made by Dedic and colleagues in the water cross maze (Dedic et al., 2018). The authors tested two groups of mice in this paradigm, one with a developmental inactivation of *Cacna1c* in forebrain excitatory neurons, and one where the gene was only targeted in adulthood. Interestingly, this difference in timing resulted in directly opposite results regarding spatial learning impairments. Radical deficits were observed in developmental knockouts, whereas the performance of adult knockout animals was on par with that of control mice, indicating that – at least concerning cognitive abilities – the presence of *Cacna1c* may be especially relevant during development.

Along the same lines, results differed depending on knockout specifics in a study conducted by Kabir and colleagues (2017a) who used a water-based Y-maze task. Mice with postnatal *Cacna1c* ablation specific to glutamatergic neurons in the forebrain showed increased escape latencies compared to WT and displayed difficulties in successfully completing the probe trials administered 1h, 24h or seven days later. These reported differences depended heavily on the deletion, as a virally induced knockout of *Cacna1c* in the adult PFC had no negative effects on Y-maze learning and animals also performed well during various probe trials post-training (Kabir et al., 2017a). In agreement with our data, no major learning deficits were detected in TS2-neo *Cacna1c* knock-in mice in other paradigms, either. Mutant and WT mice showed comparable learning abilities in a water-based Y-maze including a proximal probe trial (Bader et al., 2011) as well as when they were tested on a procedural T-maze (Kabitzke et al., 2018). Nevertheless, it needs to be mentioned that as a paradigm based on egocentric navigation, and thus the animal's understanding that it always needs to turn into one specific direction (left or right), the use of external spatial cues is not required in the latter, and task demands might therefore not be considered analogous to those of the RAM. In fact, further studies illustrate that any observed learning deficits might indeed be very task-specific, as studies using comparable knockout models to those of White (2008) and Moosmang (2005a), yet deviating paradigms, found no learning impairments during contextual fear conditioning (Kabir et al., 2017a; Langwieser et al., 2010; McKinney et al., 2008). Interestingly, an associative learning paradigm was also recently conducted in a HET rat model (Moon et al., 2020). Here, the authors discovered a prominent alteration in fear responses of HET animals to

both fear-relevant and irrelevant cues and contexts, indicating that for emotional learning paradigms, as compared to spatial learning, different result patterns may be expected in our animals.

As a HET model, the expression of *Cacna1c* in the brain of rats we examined in the RAM was of course only reduced by approximately 50% (see Study I), whereas in most of the mice with prominent spatial impairments, *Cacna1c* was fully ablated in task-relevant brain areas (Dedic et al., 2018; Kabir et al., 2017a; Moosmang et al., 2005a; White et al., 2008). This points to the notion that radical spatial learning deficits may only be apparent in animals with a full *Cacna1c* knockout, as compared to our constitutive HET approach. Following this line of reasoning, it may be assumed that instead of influencing learning behavior directly, *Cacna1c* rather influences the brain correlates underlying cognition which have been proven to be more sensitive to risk allele variance than behavioral readouts in various clinical studies (Erk et al., 2010; Thimm et al., 2011).

The hippocampus constitutes the major brain structure of interest here, as it is involved in encoding, storing and retrieving memories (Morellini, 2013). Episodic memory recall, specifically, is highly contingent on HC function (Millan et al., 2012) and patients with damage to the HC display striking declarative memory impairments (Cirillo & Seidman, 2003; Pooters et al., 2015). Moreover, spatial memory capabilities in rodents are thought be highly dependent on the HC and thus closely related to episodic memory in humans (Morellini, 2013). Not only does spatial training, for instance, increase dendritic spine density in the CA1 region of the HC (Moser et al., 1997), but HC lesions lead to disruptions in spatial memory-associated tests like the RAM (Spieker et al., 2012) and the MWM (Morris et al., 1982). In contrast to allocentric navigation, which refers to the use of spatial cues, tasks requiring egocentric navigation are less HC-dependent (Vorhees & Williams, 2014). Consistent with this, SCZ patients are impaired in allocentric versions of maze-based paradigms, but not egocentric forms (Young et al., 2009). However, even apart from spatial navigation tasks, neuropsychiatric disorders are highly associated with functional HC alterations. In ASD, abnormal hippocampal excitatory-inhibitory balance and molecular aberrations have been reported (Schafer & Schiller, 2018), and in SCZ patients, reduced HC activity has been repeatedly observed during encoding and recollection in declarative memory tasks (Dere et al., 2010; Spieker et al., 2012) and linked to recognition deficits (Schafer & Schiller, 2018). The hippocampus also appears to be deregulated in MDD (MacQueen & Frodl, 2011) while treatment via antidepressants or cognitive behavioral therapy has been shown to normalize HC activity (Schafer & Schiller, 2018). Further findings of hippocampal dysfunction in bipolar patients (Whalley et al., 2009), as well as their undiagnosed relatives, and family members of MDD or SCZ patients (Erk et al., 2014b), have led to the recognition of hippocampal abnormalities as a consistent intermediate phenotype for SCZ and major mood disorders (Erk et al., 2014b; Schafer & Schiller, 2018; van Gorp et al., 1999).

Ever since calcium channels were shown to be involved in learning processes by studies using pharmacological LTCC blockers, several avenues of research have explored the role of LTCCs and their subunits in the hippocampus, and in encoding, storage and recall of episodic memories in general (Berger & Bartsch, 2014). By now, there are multiple indications that *CACNA1C* variations contribute to disorder-associated alterations in the HC. One study, for example, investigated healthy adults and observed a trend for increased bilateral HC activity during emotional memory processing in those homozygous for the SNP rs1006737 A allele (Bigos et al., 2010). However, most other clinical research on *CACNA1C* has obtained findings of decreased regional activation (Erk et al., 2010; Erk et al., 2014a; Krug et al., 2014), as well as diminished functional coupling between the left and right HC during recall (Erk et al., 2010) and altered white matter microstructure in the HC, associated with initial learning performance in a study on healthy individuals (Dietsche et al., 2014).

In the brains of adult mice, $Ca_v1.2$ is in fact highly expressed in young mature neurons in the HC (Kabir et al., 2017c; Splawski et al., 2004). It comes as no surprise therefore, that studies in *Cacna1c* mice have put a focus on this region, and indeed, postnatal conditional hippocampus- and cortex-specific *Cacna1c* knockout models have yielded prominent impairments in learning and memory (Moosmang et al., 2005b; White et al., 2008). Within the HC, *Cacna1c* expression concentrates on the dentate gyrus (DG) (Cosgrove et al., 2017; Marschallinger et al., 2015; Sykes et al., 2018) which is heavily associated with adult neurogenesis, i.e. the survival and proliferation of new neurons (Kang et al., 2016), as well as specific forms of HC-dependent spatial learning (Deng et al., 2010; Dupret et al., 2008; Nilsson et al., 1999; Shors et al., 2002). Reduced cell proliferation, impaired neuron maturation and altered neurogenesis in general have been consistently connected to neuropsychiatric disorders (Kang et al., 2016; Moon et al., 2018) and linked to associated impairments in declarative memory (Christian et al., 2014; Millan et al., 2012). As of today, there is no definite explanation for the role of neurogenesis in disease pathophysiology, yet research in the last two decades has ascertained that *CACNA1C* appears to play a role in the mechanisms and consequences of altered neurogenesis, with many intermediate phenotypes in *Cacna1c* models being associated with this process (Kabir et al., 2016). On a molecular level, for instance, the blockade of LTCCs was found to prevent neurogenesis (Luo et al., 2005), and in conditional knockout mice, *Cacna1c* deletion caused significant neurogenesis impairments (Temme et al., 2016; Völkening et al., 2017). Initially, the effects of *Cacna1c* on neurogenesis were thought to be specific to neuron survival, with knockout animals exhibiting considerably enhanced rates of cell death by 50% (de Jesús-Cortés et al., 2016; Lee et al., 2016). However, in a more recent study using male constitutive HETs, a prominent effect on proliferation suggests *Cacna1c* is involved in neuron production as well (Moon et al., 2018).

In this context, an elegant line of experiments conducted in 2016 provides detailed insights into how *Cacna1c* may influence cognition via its selective involvement in more complex cognitive behaviors. In an effort to explain why some studies found distinct learning impairments in *Cacna1c* knockout mice, whereas others did not, the authors focused on task-specific modulators of behavioral outcomes. Mice with a CNS-specific *Cacna1c* knockout were compared to WT mice in simple and complex versions of the MWM, as well as in regular fear conditioning and context discrimination paradigms (Temme et al., 2016). Curiously, knockout animals performed on par with WT controls in the standard paradigm, yet displayed noticeable deficits in the limited cue version of the MWM where it took them significantly longer to locate the escape platform. These genotype differences were also apparent during the probe trial administered after testing. Likewise, a neuron-specific ablation of *Cacna1c* had no discernible effect on regular fear conditioning, yet strongly interfered with the animals' ability to discriminate between two contexts. Intriguingly, the completion of tasks with increased difficulty, such as the limited spatial cues version of the MWM, appears to be mediated by intact neurogenesis in the DG (Shors et al., 2002), as does the ability of context and pattern discrimination (Nakashiba et al., 2012; Saxe et al., 2006; Völkening et al., 2017). In favor of this relationship, *Cacna1c* expression was also found to be upregulated in the dentate gyrus after contextual fear conditioning (Sykes et al., 2018). The findings made by Temme and colleagues therefore suggest that *Cacna1c* may be necessary to complete demanding neurogenesis-dependent learning tests, but not for the acquisition of standard fear conditioning or the regular version of the MWM. Substantiating evidence for this theory was obtained in a significant decrease in cell proliferation and number of immature neurons in the DG by the same authors (Temme et al., 2016). Instead of being mutually exclusive, these remarks suggest that our results and those obtained in *Cacna1c* mouse studies may be jointly valid if task difficulty is taken into account. Unfortunately, there is no easy way to determine, or indeed quantify whether the RAM is less challenging than the MWM. Of course, compared to the infinite possibilities of successfully reaching the escape platform in the MWM, in the RAM an animal only needs to proceed straight ahead toward the food dish once it has made a choice for one of the eight arms on offer. Then again, instead of having one clear goal (finding the escape platform) for successful completion, rats need to employ a "win-shift" strategy *within* trials, as well as a "win-stay" approach *between* trials on the RAM until reward contingencies change, which is why the training process is often a lengthy one.

To summarize, we observed intact spatial learning abilities in male and female constitutive HET *Cacna1c* knockout rats on the RAM, with slightly inferior performance during training but stronger memory traces displayed during the probe trial on SL day 7. The discrepancies to other studies may be explained by the type of knockout employed in the respective study, as well as by task-specific demands and probe trial timing. In light of the DG-associated findings discussed in the last paragraph,

it seems reasonable to conclude that *Cacna1c* may only be required for tasks that put greater demands on cognitive abilities and only produces mild deficits during learning in partial knockouts such as ours. In this context, it appears likely that due to *Cacna1c* haploinsufficiency, neurogenesis in the DG is somewhat altered in our male HETs. However, while the notion of an altered neurogenesis in *Cacna1c* HET rats seems plausible, newly published research from our lab paints a different picture. Interestingly, in the female rats examined in this study, no effect of *Cacna1c* haploinsufficiency was observed on cell proliferation, nor on cell survival rates (Redecker et al., 2020), which begs the question whether species- and sex-specific effects (Shohayeb et al., 2018) are at play here, or if the influence of the gene on spatial learning is conveyed via mechanisms unrelated to adult neurogenesis in the DG.

Working memory

Because working memory is the basis for long-term memory and skills like e.g. language acquisition (Wallace et al., 2015), it is necessary to ensure that observed deficits in other cognitive domains are not due to basal dysfunction in working memory. However, at least in the constitutive HET *Cacna1c* rat model employed in Study III and IV, findings speak against this being an issue. As with spatial learning, working memory performance in the RAM was not strongly affected by *Cacna1c* genotype. This is in accordance with literature on *Cacna1c* mice. In two studies testing spontaneous alterations on the Y-maze in *Cacna1c* mice, effects of genotype on WM were neither observed for HET male and female mice (Zanos et al., 2015), nor for forebrain-specific knockouts (Kabir et al., 2017a). Likewise, no negative influence of *Cacna1c* on T-maze alterations was registered in mice harboring a HET knockout of the gene (Bavley et al., 2017), suggesting that reduction in *Cacna1c* expression does not selectively impair WM performance, regardless of knockout specificity.

As a matter of fact, very few working memory errors were made in general by all rat groups across learning days in Studies III and IV, which raises the question if the WM demands in the RAM were too low to detect significant differences. In human studies, differential performances in risk allele carriers have already been observed depending on working memory load and on clinical diagnosis (Hori et al., 2012; Zhang et al., 2012). It consequently seems worth investigating whether genotype differences emerge in *Cacna1c* haploinsufficient rats if the WM demand is higher, for example when there is a higher number of arms to choose from in the RAM (Vorhees & Williams, 2014).

Reversal learning

Regarding reversal learning, similar observations to spatial learning were made, in that rats of both genotypes were able to reversal learn, yet WT animals displayed a slight superiority in a steeper learning curve. This was likely owed to the fact that especially male HETs displayed perseverative tendencies on the first day of reversal learning. Nevertheless, during probe trials on RL day 7, HET animals of both sexes displayed strong memory traces of the new arm configuration.

These observations resonate well with other findings in *Cacna1c* rats, although more pronounced genotype differences were found in the same genetic manipulation in the reversal learning of a touchscreen task (Sykes et al., 2019). At first glance, our results appear inconsistent with those of Sykes and colleagues, as they found very pronounced reversal learning deficits in HET *Cacna1c* animals. However, going into the details of their findings, ours are not so dissimilar, as the reversal learning deficits in their study were mainly driven by the early phase of reversal and characterized by perseveration. Next to committing more errors during the touchscreen reversal, the rats also responded more quickly than WT initially, indicating tendencies towards impulsivity and a reduced ability to inhibit previously learned behavior. As these differences were no longer significant in the late reversal phase, this matches our results from Study III. Of note, the poor cognitive flexibility in *Cacna1c* haploinsufficient rats was also mirrored in the healthy human risk allele carriers examined in parallel, who demonstrated poorer reversal learning accuracy in a probabilistic RL task (Sykes et al., 2019).

Regarding mice, little research has been undertaken regarding cognitive flexibility. Nevertheless, constitutive TS2-neo animals showed impairments in reversal learning in two different studies, both in agreement with our data. Kabitzke and colleagues observed a trend towards impairments in male mutants during the reversal of a procedural T-maze (Kabitzke et al., 2018), and another study using a similar knock-in model found a persistence of TS2-neo mice in Y-maze reversal and when the platform was switched to a new location in the MWM (Bader et al., 2011). The Dedic study once again obtained differential effects depending on knockout timing. Mice that underwent *Cacna1c* ablation during embryonic development showed striking reversal learning impairments, whereas a deletion of *Cacna1c* during adulthood enhanced flexibility to the point where their performance surpassed that of WT controls in the water cross maze (Dedic et al., 2018). In comparison with the findings obtained by Bader (2011) and Kabitzke (2018) in TS2-neo mice, these results highlight the particular importance of *Cacna1c* in the developing forebrain for intact cognitive flexibility.

As altered neural activity and interregional connectivity have been heavily implicated in deficits of cognition (Millan et al., 2012), several clinical studies have investigated the effects of *CACNA1C* risk variants on brain anatomy and function during specific tasks and implicated the PFC and associated

frontal structures like the anterior cingulate cortex (ACC) in flexibility-related impairments. In terms of cognition, the PFC has been shown to be differentially activated when spatial rule learning is required to complete a task (Miller, 2000), and has been linked to memory encoding and retrieval in various species (Braver et al., 2001; Khader et al., 2016; Pooters et al., 2015). Most importantly, however, the PFC is ideally suited to exert top-down control on other brain regions due to its high level of connectivity (Miller, 2000), and thus it is especially relevant for executive function (Brady & Floresco, 2015; Manoach, 2016), like cognitive flexibility (Keeler & Robbins, 2011) and reversal learning (Sykes et al., 2019; Zhang et al., 2018a), as well as for working memory in humans (Gilmartin et al., 2013; Miller, 2000) and rodents (Morellini, 2013).

In mental disorders, abnormally reduced PFC activity has been noted for MDD and SCZ patients during memory encoding and retrieval (Barch & Ceaser, 2012; Dere et al., 2010; Krug et al., 2014) and paradigms requiring executive function (Berrettini, 2005; Zhang et al., 2019). Interestingly, this pattern of altered activation is considered to be a sign of reduced working memory efficiency (Backes et al., 2014; Manoach, 2016) and can be observed even in close relatives of SCZ (Barch & Ceaser, 2012; Callicott et al., 2003) and other at-risk individuals (Powell et al., 2015). PFC dysfunction has also been observed in ASD (Grady & Keightley, 2002; Pu et al., 2019) and linked to aberrant social stimuli processing in MDD and psychotic disorders (Grady & Keightley, 2002; Porcelli et al., 2019). Next to altered PFC activity in social cue processing (Dima et al., 2013; Jogia et al., 2011; Radua et al., 2013), risk variants of *CACNA1C* have also been strongly associated with differential effects on PFC activity during cognitive paradigms. For instance, increased PFC activity was seen in risk allele carriers among healthy participants during various executive function tasks (Bigos et al., 2010; Krug et al., 2010), whereas the opposite was true for the PFC in working memory efforts (Paulus et al., 2014), as well as for the executive control of attention in the medial frontal lobe (Thimm et al., 2011). During semantic verbal fluency paradigms, significantly increased activity in the PFC was found in the first study on *CACNA1C*-associated brain activation in MDD patients (Backes et al., 2014), along with a similar trend in SCZ and BPD subjects (Tecelão et al., 2019). In contrast, reduced activation in the left dorsolateral PFC was observed during memory encoding (Erk et al., 2014a; Krug et al., 2014). As a candidate mechanism of $Ca_v1.2$ action in the human PFC, alterations in the synaptic inhibitory-excitatory balance have been postulated (Kabir et al., 2017a).

Considered to be involved in the selection of remote spatial memories (Teixeira et al., 2006), the inhibition of irrelevant information during memory recall (Nieuwenhuis & Takashima, 2011) and response monitoring in general (Manoach, 2016), the ACC, as another important frontal brain region, has been similarly implicated. ACC dysfunction has been reported in MDD, BPD and SCZ patients, as well as in their relatives, and is exacerbated in individuals carrying the rs1006737 risk allele (Erk et al.,

2010; Erk et al., 2014b). On a structural level, Wang and colleagues ascertained that risk allele carriers have bilaterally increased gray matter density in the ACC and PFC (Wang et al., 2011) and in another study, a different intronic risk allele was associated with increased cortical surface area of the dorsolateral PFC, but not thickness in SCZ patients, while the opposite was true for healthy controls (Zheng et al., 2016). Similarly, the importance of *Cacna1c* expression in the forebrain for intact learning has been highlighted by relevant mouse studies (Dedic et al., 2018; Kabir et al., 2017a; Moosmang et al., 2005a; White et al., 2008) and together, these findings from clinical and preclinical research emphasize the relevance of *CACNA1C* in the frontal lobe as an essential anatomical structure for reversal learning, working memory and executive function.

In conclusion, HET *Cacna1c* rats were slightly retarded in terms of cognitive flexibility, but then displayed an even stronger memory trace than WT controls after seven days of improving their reversal learning performance. Regarding associated brain structures and potentially mediating mechanisms, *Cacna1c* appears to be particularly relevant in the developing forebrain to ensure cognitive flexibility in adulthood.

Reward sensitivity

There were no major differences detected in reward sensitivity, which we controlled for because maze performance can be influenced by motivation and reward size (Crespi; Görisch & Schwarting, 2006) and *CACNA1C* has been shown to alter the behavioral responses to reward (Lancaster et al., 2014) and affect brain activity during reward processing in human risk allele carriers (Wessa et al., 2010). In addition, the results from Study I and II suggest that approach behavior towards social reward is significantly altered in *Cacna1c* HET rats.

However, all groups proved to be reward sensitive on the RAM by preferably entering the highly rewarded arm rather than the less rewarded arms during the two probe trials administered after spatial and reversal learning. Consequently, motivational factors likely do not account for the observed genotype differences in spatial learning, reversal and cognitive flexibility. This observation is paralleled by findings in a human cohort tested in a probabilistic reversal learning task where performance differences in human subjects carrying a *CACNA1C* risk allele were not associated with task motivation either (Sykes et al., 2019). Hence, the intact reward sensitivity displayed by rats with constitutive haploinsufficiency during RAM training suggests that the altered reward processing observed in our USV & playback studies is specific to rewards that are social in nature and does not extend to food reward-based learning paradigms.

Notably, however, *Cacna1c* mice have been suspected to employ different search strategies than WT controls (Koppe et al., 2017; Moosmang et al., 2005a). When tested in a labyrinth maze, forebrain-specific *Cacna1c* knockout mice displayed less efficient strategies in significantly longer path lengths to the maze exit (Moosmang et al., 2005a), although this genotype difference was no longer apparent in probe trials conducted 10 or 17 days after training. Koppe and colleagues hypothesized that *Cacna1c* animals, instead of learning a cue-reward association as intended, rather based their decisions in a cue-discrimination learning paradigm on where a reward was located previously (Koppe et al., 2017). Reward processing, and especially the learning of cue-reward associations is associated with the PFC (Miller, 2000; Ragozzino et al., 1999), and as already discussed, animals with regional inactivation of *Cacna1c* in the forebrain have displayed prominent behavioral alterations in various paradigms (Dedic et al., 2018; Kabir et al., 2017a; Lee et al., 2012; Moosmang et al., 2005a; White et al., 2008). Interestingly, as reversal learning requires the acquisition of a new cue-reward contingency in addition to the inhibition of previously learnt associations (Quan et al., 2010), the employment of an outcome-based learning strategy might help explain the perseverative tendencies observed in the HET rats of Study III.

Novel object recognition

The performance in the novel object recognition test was found not to be affected by genotype or sex in Study III and IV. Both HET rats, as well as their WT siblings, did not differ in their exploratory behavior in the first trial and were able to successfully distinguish between two objects after an inter-trial interval (ITI) of 30 minutes, demonstrated by more time spent sniffing the novel object compared to the familiar one.

Initially, these results seem incongruent to those obtained in constitutive *Cacna1c* HET mice (Zanos et al., 2015). Zanos and colleagues investigated *Cacna1c* effects on cognition across the lifespan and employed the NOR paradigm with almost identical specifications yet obtained significant genotype differences in the ability to distinguish novel and familiar objects, which stands in direct contrast to the results from Study III. However, looking at the details, one finds that the genotype difference observed in their experiment was driven by the older group of mice at about 17–18 months. By comparing the age of mice (Dutta & Sengupta, 2016) and rats (Andreollo et al., 2012), it becomes clear that the rats used in Study III were still comparatively young at three months and thus more equivalent in age to the group of young mice used by Zanos and colleagues. Those animals, in fact, displayed intact novel object recognition, regardless of genotype or sex, which matches our results. Further evidence that this reasoning is correct can be found in a study using mice with a conditional knockout of *Cacna1c* in the ACC and, likewise, used comparatively young animals that displayed NOR abilities on par with

controls (Jeon et al., 2010), thus negating a prominent impact of *Cacna1c* on object recognition in young rodents.

Then again, the results obtained in aged mice suggest that *Cacna1c* haploinsufficiency might have a sex-dependent protective influence on object memory later in life (Zanos et al., 2015) when cognitive function, and NOR performance in particular is known to deteriorate (Young et al., 2009). This has not been investigated in rats so far. It is furthermore not clear, whether this effect may occur due to e.g. differential habituation effects due to *Cacna1c* ablation (Bader et al., 2011; Gaskin et al., 2010) or if an explanation may be found in the molecular changes associated with ageing. Indeed, ageing mice exhibit an increase of hippocampal *Cacna1c* mRNA and LTCC current densities in HC neurons, in addition to memory deficits (Zanos et al., 2015). By contrast, the hippocampus of *Cacna1c* HET mice does not show such a substantial increase of *Cacna1c* mRNA expression compared to WT, and as these animals are also less impaired regarding novel object recognition later in life, this suggests that higher levels of *Cacna1c* in the hippocampus have a detrimental effect on learning abilities (Zanos et al., 2015). This notion is substantiated by the fact that several studies have shown LTCC blockers to attenuate the age-associated decline of cognitive abilities in aged rodents (Berger & Bartsch, 2014; Ingram et al., 1994). In vitro findings regarding neuron survival furthermore support the idea that a reduction of intracellular calcium may have a protective effect on cognitive abilities, as an elevated calcium influx and LTCC density are connected to age-related cell death (Disterhoft et al., 1994; Porter et al., 1997) and the rate of neurogenesis is known to deteriorate in older rats (Kang et al., 2016; Kuhn et al., 1996).

Coming back to the results obtained in Study III, these data argue for a loss of function in the calcium channels of our HET *Cacna1c* rats, which may contribute to a potentially protective effect on long-term learning. A longitudinal study of *Cacna1c* haploinsufficient rats in terms of RAM and NOR performance is warranted, combined with measures of neurogenesis to see if the reduced *Cacna1c* expression indeed proves to preserve learning abilities in aged animals across species and paradigms, and to determine whether this mechanism is mediated by the hippocampus.

Sex differences in cognition

In addition to the increased efficiency during spatial learning, we observed a minor enhancement of cognitive flexibility in female HETs on RL1, which did not diminish their robust memory trace during the probe trial. Counterintuitively, female rats are not considered to be superior to males in terms of cognitive flexibility in general – in fact, quite the opposite is true (Schoenberg et al., 2019; Westbrook et al., 2018). Seeing that in the males of Study III, the relationship of genotype to flexibility was almost vice versa, and cognitive symptoms in neuropsychiatric disorders are usually more pronounced in

males (Burrows et al., 2011), these findings raise the question of how alterations in *Cacna1c* expression levels exert a sex-dependent effect on cognition.

The observed differences could, for instance, be due to differing critical time periods for the development of learning-associated brain structures in males and females. As outlined in the section on reversal learning, *Cacna1c* appears to be particularly relevant for cognitive abilities during development, which is an important point in time for sex-specific differentiation of the rodent brain (Lenz et al., 2012). Interestingly, this notion also finds support in a clinical study investigating various psychological and neurobiological measures in a large human cohort. During a cognitive task, reduced activity was observed in the PFC and hippocampus of female risk allele carriers, but not in men (Takeuchi et al., 2018), and the PFC is known to be quite vulnerable for estrogen depletion in humans, as well as in rats (McEwen & Morrison, 2013). This is in line with my previous comments on the importance of the PFC and hippocampus, and the frontolimbic system in general in mediating *CACNA1C* effects.

Unfortunately, most of the existing studies on learning and memory effects of *Cacna1c* in rodents examined males only (Bader et al., 2011; Dedic et al., 2018; Jeon et al., 2010; Kabir et al., 2017a; Kabitzke et al., 2018; Terrillion et al., 2017b), and those that included females pooled data from both sexes (Temme et al., 2016; White et al., 2008). However, there are indications that *Cacna1c* may modulate behaviors other than cognition in a sex-dependent manner as well. For example, Dao and colleagues reported a more resilient phenotype in *Cacna1c* females, including diminished startle response, decreased learned helplessness and an attenuated hyperlocomotor response to amphetamine (Dao et al., 2010). Differential findings were also observed between male and female animals in terms of anxiety (Dao et al., 2010; Lee et al., 2012). In terms of potential reasons, it was hypothesized that *Cacna1c* gene products may be dissimilarly expressed in male and female individuals (Dao et al., 2010). However, as we and another more recent study have ascertained, this is not the case (see Study I and Zanos et al., 2015). It is conceivable that *Cacna1c* interacts with gonadal hormones like estradiol, which directly potentiates LTCCs in vitro (Sarkar et al., 2008) and is linked to LTCC-associated neuroprotective effects on cell death in vivo (Sribnick et al., 2009). Furthermore, estradiol has been shown to prevent age-related $Ca_v1.2$ increase in the HC of female rats (Brewer et al., 2009). In the same vein, *Cacna1c* is associated with depressive behaviors (Dao et al., 2010), which may be modulated in a sex-specific manner by estrogen, as evidenced in the association of low estrogen levels and greater depressive-like behavior in rodents (Hajszan et al., 2010). This latter notion also supports results obtained regarding the prevalence of MDD, which in women is almost twice as high as in men (Otte et al., 2016).

Together, the experiments in Study III and IV paint the effects of alterations in *Cacna1c* on cognitive abilities in a somewhat more positive light than comparable experiments on mice and indicate that basal cognitive functioning is intact in rats carrying *Cacna1c* on only one allele. Even though heterozygosity led to impaired cognitive flexibility in males specifically, our results hint at a beneficial effect of *Cacna1c* haploinsufficiency on long-term learning. While our evidence is in line with the hypothesis that brain structures like the PFC, ACC and hippocampus mediate *Cacna1c* effects on behavior, however, the task-dependent, knockout-specific and age-related mechanisms resulting in differential performances in male and female HET rodents require further investigation. Only then will we be able to pinpoint how exactly *Cacna1c* affects intermediate cognitive phenotypes of relevance to neuropsychiatric disorders like MDD, BPD, SCZ and ASD.

Influence of environmental factors on cognition

Next to genetic variation, environmental risk and protective factors are known to influence the development and progression of neuropsychiatric disorders (Alloy et al., 2005; Cirillo & Seidman, 2003; DiLalla et al., 2017; Mullins & Lewis, 2017), as well as the cognitive dysfunction often associated with a diagnosis of the same (Pugliese et al., 2019; Short & Baram, 2019).

In rats, the post-weaning period is considered a crucial time window for future development, and manipulations of housing conditions in this phase are an established approach to elicit behavioral changes later in life (Burrows & Hannan, 2016). Based on previous findings in animals raised in social isolation or enriched environments, we expected ISO to negatively affect measures of learning and flexibility (Amitai et al., 2014; Lapiz et al., 2003; Vorhees & Williams, 2014), as well as beneficial effects on memory by ENR (Nithianantharajah & Hannan, 2006; van Praag et al., 2000).

Spatial learning

The four weeks spent in environmental ENR exerted an overall positive influence on spatial learning abilities, consistently resulting in fewer erroneous arm entries for rats raised in ENR. These results are consistent with numerous research papers on the positive effects of ENR on learning. Social isolation, on the other hand, had a slightly negative influence on spatial learning. No significant reduction of either errors or time-to-criterion was observed from the first day of spatial learning to the last, yet in the SL probe trial administered on day 7, ISO animals displayed a sound performance in both genotypes, indicative of intact spatial learning abilities in general.

Improved spatial learning due to environmental ENR is one of the most consistent findings in the literature on ENR (van Praag et al., 2000). While there are many papers concerned with ENR effects on

spatial learning in mice (Bennett et al., 2006; Garthe et al., 2016; Hendershott et al., 2016), I will focus mostly on rat studies here, as they are of higher relevance to the model used in Study IV.

Construct	Paradigm	ISO	ENR
Spatial Learning	Radial arm maze SL	– no improvement in error rates – no improvement in time-to-criterion	+ fewer errors in general
Working Memory	Radial arm maze SL+RL	+ fewer WM errors over time	NE
Reversal Learning	Radial arm maze RL	NE	+ higher flexibility on RL1 + fewer errors in general
Reward sensitivity	Radial arm maze SL+RL	NE	NE
Object recognition	Novel object recognition	– no preference for novel object	NE

Table 7: Summary of key findings: Environmental effects on cognition in Study IV. Effects always refer to the comparison with the other two housing conditions. – = negative effect of environment on performance. + = positive effect of environment on performance. NE = no effect. ENR = enrichment. ISO = social isolation. SL = spatial learning. RL = reversal learning. WM = working memory.

Several studies have already investigated the effects of ENR on the MWM performance in rats and observed enhanced learning capabilities compared to STD-housed control animals. Even though there was, for instance, no marked difference between STD and ENR on the first training day in one experiment, enriched rats subsequently showed significantly faster acquisition of platform locations in the MWM, as well as an augmented memory trace in the following probe trial (Schrijver et al., 2002). In general, faster escape latencies have not only been consistently observed in enriched rats subjected to the MWM (Simpson & Kelly, 2011) but also in other paradigms, such as the water maze test (Nilsson et al., 1999). Interestingly, it appears that the physical aspect of ENR in particular is essential for the promotion of cognitive abilities, as compared to social factors. In one study conducted in our lab, learning was significantly enhanced in physically enriched rats, whereas the social aspect only provided a minor additional benefit for performance in the spatial object recognition paradigm (Brenes et al., 2016). Importantly, this effect was not due to increased exercise on a running wheel, which was precluded from the ENR setup as physical activity is known to have a positive influence on cognition in and of itself (Nithianantharajah & Hannan, 2006; van Praag et al., 2000). The relevance of physical ENR was further highlighted in another study that showed spatial learning was improved irrespective of the social background in housing (Schrijver et al., 2002). However, as ENR had no beneficial effect on RAM performance in animals that were merely exposed to physical, but not social ENR (Brillaud et al., 2005), the additional positive effect of the social ENR in our study may not be completely disregarded.

Notably, the finding of attenuated spatial learning performance by enriched rodents extends to both the MWM and RAM. In fact, the animals in another study displayed an almost identical learning pattern as the rats in Study IV, in that the ENR group made fewer errors during learning, but both standard and enriched rats were able to perform well on the RAM (Leggio et al., 2005). As a matter of fact, this result,

in combination with the accelerated MWM acquisition in their enriched subjects, prompted the authors to hypothesize that ENR does not affect learning directly, but rather facilitates the speed of knowledge acquisition by enabling rats to process spatial data more efficiently, indicating that task-relevant information is transferred to long-term memory in an accelerated manner in these animals (Leggio et al., 2005; Schrijver et al., 2002).

As a potential neural correlate of this behavioral observation, the facilitation of long-term potentiation (LTP) in the HC via environmental ENR seems to be the most plausible pathway. The LTP process is an experience-dependent form of synaptic plasticity (Bauer et al., 2002) and refers to the sustained increase of synaptic strength (Millan et al., 2012). In the hippocampus, these long-lasting synaptic changes are a well-established candidate mechanism for spatial learning (Isaac et al., 2009; Shapiro, 2001) and memory consolidation, i.e. the transfer from short-term storage into long-term memory, is thought to be heavily dependent on intact LTP (Bliss & Collingridge, 1993; Martin et al., 2000; Wang & Morris, 2010). The positive impact of ENR on hippocampus-dependent tasks is one of the most reliable finding in research on the consequences of ENR (van Praag et al., 2000). This makes perfect sense, as the HC is known to be very sensitive to environmental influence (Kempermann et al., 1997), and the development of the dentate gyrus & hippocampus in general is thought to peak at PND 20-30 and to progress until PND 35-40 (Lapiz et al., 2003), coinciding with the post-weaning phase and suggesting that environmental manipulations may be particularly effective during this time. In terms of synaptic plasticity, ENR has been consistently linked to the promotion of LTP over the last two decades (Nithianantharajah & Hannan, 2006; van Praag et al., 2000), and in rats, stable LTP enhancement in the CA1 region of the HC has been observed after the exposure to several weeks of continuous ENR (Duffy et al., 2001), a finding that has been replicated with success using various ENR durations (Artola et al., 2006; Cui et al., 2006) and found in hippocampal slices in vitro, as well (Green & Greenough, 1986). Conversely, LTP alterations have been related to cognitive dysfunction (Millan et al., 2012) and synaptic plasticity is known to be significantly impaired in patients suffering from BPD (Woodside et al., 2004) and ASD (Chaste & Leboyer, 2012), highlighting the potential role of LTP alterations in disease etiology. On top of hippocampal LTP, ENR is also known to promote adult neurogenesis in the dentate gyrus, especially in terms of reduced apoptotic cell death (Nithianantharajah & Hannan, 2006; van Praag et al., 2000). As with the positive effects on cognitive behavior, physical, more so than social ENR was found to upregulate hippocampal neurogenesis (Brenes et al., 2016), and improved spatial and non-spatial learning abilities in rats were found to be concomitant with increases in neurogenesis in the adult rat DG in several studies (Bruel-Jungerman et al., 2005; Nilsson et al., 1999). These neurogenesis-related findings do not preclude LTP as a potential underlying mechanism, though. Indeed, in one study where Sprague-Dawley rats were raised in standardized ENR cages, animals later displayed enhanced

LTP in addition to elevated hippocampal neurogenesis, and accompanied by improved spatial learning and cognitive flexibility (Fares et al., 2013).

In addition to these cell process-specific theories, another explanation for the beneficial effects of ENR in maze paradigms may be that it diminishes anxiety. As ENR improved performance in rats subjected to the water-based RAM in terms of error rates and direct approach to the goal platform, yet did not alter activity in the dorsal hippocampus, which is usually associated with spatial learning and ENR (Moser et al., 1993; Zhang et al., 2018b), several authors conjectured that instead of affecting learning directly, ENR ameliorated potential anxiety phenotypes in these rodents (Sampedro-Piquero et al., 2013). This theory finds support in a study showing anxiety was reduced alongside better spatial learning abilities via ENR in rats (Fares et al., 2013). However, this theory does not stand in contrast to e.g. postulated effects of ENR on neurogenesis, as this, in fact, has been shown to decrease anxiety in and of itself. When adult hippocampal neurogenesis is increased pharmacologically or via exercise, for example, the display of anxiety in treated rodents is significantly reduced (Kang et al., 2016). It is conceivable therefore, that ENR – in addition to promoting spatial learning via neurogenesis and LTP – also has an anxiolytic effect on rodents that can improve maze performance.

Regarding effects of negative environmental factors, the slight deficit induced by ISO during training did not lead to these rats failing the spatial learning probe trial in Study IV. This adds to the mixed evidence concerning negative consequences of social isolation on spatial learning in the existing literature (Fone & Porkess, 2008). No specific difference, for instance, was observed between ISO and STD rats in the RAM (Schrijver & Würbel, 2001), nor in mice performing on the Y-maze (Ouchi et al., 2013). Another study even proposed that isolation enhanced spatial learning in the MWM (Wongwitdecha & Marsden, 1996), although these authors only used escape latency as a dependent variable and not path length, which is considered to be the more accurate measure, as it does not depend on e.g. swim speed and phases of immobility (Young et al., 2009). Then again, more prominent deficits compared to STD animals may have been elicited in our experiments after an additional month of ISO (Quan et al., 2010), even though on the flip side, difficulties during spatial learning in the Morris water maze have already been observed after just three weeks spent in isolation (Wade & Maier, 1986).

In general, our ISO results from Study IV argue in favor of the notion that effects of environmental stress on spatial learning is not straightforward (Vorhees & Williams, 2014). Compared to the mild ISO effects, however, we found a robust positive effect of ENR on spatial learning, in accordance with previous research and most likely mediated via neuronal mechanisms in the hippocampus. In the past, doubts about the long-term effects of ENR were voiced in the much-cited review by van Praag, based

on the disappearance of positive effects on MWM performance after test animals were group-housed for a certain amount of time (van Praag et al., 2000). In direct contrast of this claim, though, we show that even after almost 10 weeks of standard housing, the rats tested on the radial arm maze in Study IV still profit considerably from one month of post-weaning ENR.

Working memory

As stated above, only a small number of working memory errors were committed in general. In fact, an improvement of WM errors was only observed in ISO groups, which is in accordance with another paper investigating RAM performance in isolated rats and found improved WM compared to socially housed rats (Grigoryan et al., 2010). However, looking at the descriptive data in Study IV, this finding, rather than owing to positive influence of ISO on working memory, is more likely due to the fact that isolates committed more initial WM errors relative to the other groups – a behavior they improved over time. This, too, finds corroboration in the literature on male rats isolated in the third postnatal week and tested on the 12-arm radial maze at the age of three months (Sandstrom & Hart, 2005). These animals were also found to commit a higher number of spatial working memory errors than controls in the initial training days but were able to adjust their performance to comparable levels across several days.

The brain structure most heavily associated with working memory is the prefrontal cortex (Baddeley, 1992), as it is the only cortical structure so far identified to reliably link events separated by time and to keep items in a short-term buffer across distracting stimuli (Gilmartin et al., 2013; Miller, 2000). Notably, aberrant PFC activation during working memory tasks is usually observed in patients with SCZ and their relatives (Callicott et al., 2003; Manoach, 2016), as well as in MDD and ASD (Grady & Keightley, 2002; Pu et al., 2019). Hence, the alterations caused in the PFC by social isolation rearing (Baarendse et al., 2013; Fone & Porkess, 2008) may contribute to an initial difficulty for ISO rats to refrain from entering previously visited arms on the RAM. However, the isolates in Study IV apparently recovered from this initial handicap, as displayed in the significant reduction in WM errors. Looking at potential explanations, compensatory mechanisms via plasticity processes in the PFC after external insults have already been investigated but are not fully understood to date (McEwen & Morrison, 2013; Müller et al., 2002; Voytek et al., 2010).

Reversal learning

All groups were able to learn the new configuration of rewarded arms over seven training days. At the same time, cognitive flexibility on RL1 was found to be augmented by environmental ENR. Both genotypes previously housed in ENR conditions showed ideal patterns of cognitive flexibility, which

was not observed so consistently in any other housing condition. In the same vein, ENR also enhanced reversal learning performance in Study IV, and in two studies testing enriched mice in the MWM (Garthe et al., 2016; Hendershott et al., 2016). It is believed that environmental ENR enables rodents to adapt more quickly to novel situations (Larsson et al., 2002) and to augment their ability to rapidly shift between search strategies at the change of reward contingencies on the RAM (Leggio et al., 2005). In fact, ENR has been invariably associated with improved cognitive flexibility and the rescue of executive function-associated impairments in translational rodent models for neurodegenerative disorders (Gelfo, 2019).

These behavioral observations are paralleled by a direct ENR-induced enhancement of neurogenesis, as already outlined, and a positive contribution of ENR to PFC- and HC-associated molecules involved in neuronal development, such as BDNF (brain-derived neurotrophic factor). BDNF is expressed throughout the rodent brain, but can be found in especially high concentrations in the PFC and hippocampus (Savitz et al., 2006), both of which are essential for performance on the RAM (Spieker et al., 2012). It is believed to play a key role in several behaviors related to spatial learning and memory, as well as cognition in general (Costa et al., 2015). Interestingly, the expression of BDNF is known to be altered in mood disorders (Dunham et al., 2009), and the effect of antidepressants supposedly depends heavily on intact BDNF signaling (Costa et al., 2015). Furthermore, BDNF mediates other processes associated with learning performance, as it is an important regulator of synaptic plasticity in the hippocampal formation and cerebral cortex (Cunha et al., 2010; Korte et al., 1998; Novkovic et al., 2015) and in addition, the infusion of BDNF into the adult rat HC enhances cell proliferation and neurogenesis (Scharfman et al., 2005). In a study investigating the effects of a *BDNF* polymorphism on cognitive flexibility in humans, differential tendencies for perseverative errors in the Wisconsin Card Sorting Test were observed, depending on *BDNF* gene variant (Gabrys et al., 2017). In rodents, too, the lack of BDNF was specifically connected to prominent reversal learning deficits and altered synaptic plasticity in the PFC, whereas spatial memory per se was not affected (Amodeo et al., 2017; Sakata et al., 2013), indicating a particularly important role for the absence of BDNF in cognitive flexibility impairments. By contrast, exogenous BDNF administration was able to promote strategy shifting in mice by minimizing response perseveration (D'Amore et al., 2013). In terms of environmental ENR, the elevation of BDNF levels is considered a trademark effect of exposure to ENR (Ickes et al., 2000). Post-weaning ENR has been shown to augment BDNF in the hippocampus of adult rats (Mosaferi et al., 2015) alongside improved cognitive flexibility after maternal deprivation (Menezes et al., 2020). Compared to age-matched controls, rats that undergo ENR and perform better in spatial memory tasks additionally exhibit long-term increased BDNF levels not only in the HC (Falkenberg et al., 1992) but also in the cortex and basal forebrain (Ickes et al., 2000). In fact, the upregulation of BDNF has been

suggested to mediate enhanced neurogenesis in the HC of animals reared in ENR following weaning (Rossi et al., 2006). As childhood and adolescence are considered to be critical time windows for the development of cognitive flexibility (Chelune & Baer, 1986; Dick, 2014), it seems conceivable that environmental stimuli affecting flexibility-associated BDNF levels would be especially impactful in this vital period of cognitive development.

Contrary to ENR, one would expect striking impairments in the socially isolated rats of Study IV, as sensory stimulation and exposure to unexpected events is known to be drastically reduced during post-weaning social isolation (Seffer et al., 2015), and ISO is generally known to influence behavior associated with cognitive flexibility, like fear extinction, reversal learning and attentional set shifting (Fone & Porkess, 2008). However, we observed no remarkable effects of ISO housing on reversal learning or flexibility measures, and instead noted strong memory traces in isolates after reversal learning.

Apart from our results in Study IV, other authors have found contradictory effects of ISO in a display of enhanced reversal learning in the MWM (Wongwitdecha & Marsden, 1996), but also retardation in non-spatial flexibility, such as a slow adjustment of strategy in a paradigm rewarding nose pokes with milkshake, a correct lever press with sucrose, or during a two-choice digging-based task (Amitai et al., 2014; Jones et al., 1991; Powell et al., 2015). All of these three studies found pronounced reversal learning deficits in isolated animals, yet in contrast to our testing protocols, either tested the animals after a prolonged period of experimental housing (Amitai et al., 2014), used female rats (Jones et al., 1991), or compared animals with each other that were still living in social isolation (Powell et al., 2015). Together, these results suggest that prominent impairments in reversal learning and cognitive flexibility in ISO rats may only be observed after longer isolation periods or shorter intervals until the start of testing, and that deficits may be mitigated by passing time and potentially compensatory processes. The stated contradictions furthermore suggest that the impact of ISO on reversal learning may be task- and sex-dependent, which fits in well with the fact that no effect of ISO on the ability of male rats to reversal learn the RAM, specifically, was found by other authors (Schrijver & Würbel, 2001), as well as in Study IV. The inconsistency of these results may also be explained by the notion that ISO, rather than influencing reversal learning itself, may affect underlying neurobiological structures like the frontolimbic pathway (Fone & Porkess, 2008). In fact, Würbel proposed the theory that social isolation may not impair response inhibition directly, but rather exert its influence indirectly via the attentional selection of responses, leading to a deficit in allocating mental resources to the correct course of action, but generally intact spatial learning (Birrell & Brown, 2000; Schrijver & Würbel, 2001; Würbel, 2001).

In contrast to the lack of a consistent ISO influence on reversal learning, however, we did observe a significant beneficial effect of ENR on both genotypes, promoting cognitive flexibility and reversal learning and likely mediated by an upregulation of BDNF. Interestingly, rats reared in barren cages do not show reversal learning impairments (Rose et al., 1988), indicating that while exerting a positive influence on performance in spatial mazes (Brenes et al., 2016; Schrijver et al., 2002), the physical aspects of ENR may not be essential for intact reversal learning in rats, which is mirrored by the results from Study III and IV.

Reward sensitivity

It has previously been proposed that animals reared in social isolation are differentially incentivized by reward than socially housed animals (Rose et al., 1986), and to show increased reward sensitivity (Jones et al., 1990), especially toward food rewards (Morgan, 1975). Nevertheless, reward sensitivity was found to be generally intact in all three housing conditions. Descriptively, ENR rats appeared the least sensitive to reward in the reversal learning probe, which mirrors the general consensus that ENR animals exhibit poorer reward discrimination (Kirkpatrick et al., 2013; van der Harst et al., 2003; Xu et al., 2007) and are less susceptible to the addictive effects of psychostimulants (Nithianantharajah & Hannan, 2006).

Novel object recognition

While novel object recognition was found to be intact in animals previously housed in STD or ENR conditions, social isolation had a consistent negative effect on NOR abilities. Whereas all other groups were able to perform well, both WT and HET genotypes failed to significantly distinguish between novel and familiar object under ISO after an ITI of 30 minutes. These observations resonate well with the general theory that social isolation impairs object recognition abilities. At similar exploration rates in trial 1, isolated animals were found to fail recognition testing in trial 2 directly after ISO (Bianchi et al., 2006; McIntosh et al., 2013), as well as with a delay between the end of social isolation and the implementation of NOR (Valluy et al., 2015). In fact, Study IV was able to replicate the findings of this latter experiment that still found impaired NOR abilities six weeks after isolation had ended. Indeed, the negative influence of ISO on object recognition is so strong, that it can also be induced if animals are subjected to ISO outside of the critical time window in juvenility (Fischer et al., 2012), although sex differences appear to influence the results, as females are able to distinguish novel and familiar object up to an ITI of one hour (McLean et al., 2010). Conversely, a more recent study found no effects of isolation on NOR abilities in male rats, yet contrary to common practice they only counted the number of interactions between rat and item, instead of the duration of object interaction. On top of this, their ITI was quite short at five minutes (Templer et al., 2018) which conforms to the demonstration by

McLean and colleagues that the effects of ISO appear to be more aggravating with longer ITIs (McLean et al., 2010).

A likely candidate mechanism that may mediate the effects of social isolation on NOR capabilities is the dopamine-signaling within the prefrontal cortex. Watson and colleagues obtained evidence that the selective blockade of DA-signaling in the PFC can induce impairments of object recognition memory in rats (Watson et al., 2012), whereas social isolation can lead to cellular and synaptic changes and a loss of sensitivity to dopamine in neurons of the medial PFC (Baarendse et al., 2013). In fact, a recent review focused on the long-term attenuating effects of ISO on the basal DA metabolism in the rodent prefrontal cortex (Walker et al., 2019), highlighting the structure's potential involvement in NOR impairments.

By comparison, the PFC is usually affected by social isolation to a greater extent than the hippocampus (Fone & Porkess, 2008). However, ISO-induced NOR deficits in rats have been shown to be accompanied by structural alterations in the HC (Bianchi et al., 2006), and temporal lobe damage has likewise been shown to diminish recognition abilities in human subjects (Wallace et al., 2015). The HC is known to be quite sensitive to stress (Kempermann et al., 1997; Sandi & Haller, 2015) and it is therefore no surprise that in humans, early life adversity such as childhood neglect is closely linked to hippocampal alterations (MacQueen & Frodl, 2011; Millan et al., 2012). In rats, juvenile social deprivation has been associated with decreased density of dendritic spines and plasticity in the HC (Fone & Porkess, 2008), as well as reduced dorsal HC volume and deficits in HC-dependent memory (Short & Baram, 2019). Then again, the reliance of NOR abilities on the hippocampus has been called into question, as recognition does not appear to be particularly sensitive to hippocampal damage if exploration and test trial occur in the same context (Keeler & Robbins, 2011; O'Brien et al., 2006; Young et al., 2009). However, the assumption has been made that performance in this paradigm may depend on intact neurogenesis in the hippocampus "under certain circumstances" (Bruel-Jungerman et al., 2005, p. 519) and that the paradigm can be modified to increase or decrease this dependence (Dere et al., 2007), e.g. by using complex vs. simple objects (Hodges, 1996). This is in agreement with the idea raised in a previous section of this dissertation, suggesting hippocampal neurogenesis may only be required for tasks which are more challenging (Shors et al., 2002). Hence, the negative influence of social isolation on neurogenesis (Lu, 2003) may cause NOR performance to deteriorate.

Contrarily, social and physical ENR had no effect on NOR abilities in Study IV, which is in accordance with the existing literature on rats and mice (Bruel-Jungerman et al., 2005; Melani et al., 2017; Schrijver et al., 2002). One of these studies found that enriched animals display even less of a preference for the novel object in a paradigm set-up involving short ITIs (Schrijver et al., 2002). Taking the previously

addressed idea into account that enriched animals process information more quickly and have an accelerated habituation to novelty (Leggio et al., 2005), it seems conceivable that a simple novel object would not be of great interest to those groups with a vast experience in physically enriched cages. Then again, task-specific modifications do appear to be able to tease out a recognition advantage in enriched animals if the demands on cognitive abilities are greater, e.g. at ITIs longer than one hour (Bruel-Jungerman et al., 2005; Melani et al., 2017), if the period of ENR is prolonged (Hullinger et al., 2015), or if ENR is provided to strongly impaired animals in the sense of a rescue by ENR (Rampon et al., 2000).

Concluding the discussion on the environmental effect on cognition, we obtained evidence for a prominent beneficial effect of environmental ENR on spatial, but especially reversal learning and cognitive flexibility, potentially mediated via neurogenesis or BDNF. By contrast, social isolation only caused a slight attenuation in spatial learning and working memory measures, yet a significant impairment of NOR abilities in the rats of Study IV. ENR had no impact on object recognition, although paradigms using a longer ITI may yield different results. Most importantly, we were able to show that the positive and detrimental effects of post-weaning environmental manipulation were evident in adult rats housed under standard conditions for several weeks after the exposure to ENR and ISO, respectively, had ended.

Interaction of gene x environment in cognition

In terms of GxE interactions in cognitive paradigms, we observed *Cacna1c*-modulated effects for both ISO and ENR. Unlike WT siblings, for instance, HET animals were negatively influenced by isolation and – in contrast to all five other groups – did not show a significant improvement of error rates on the last day of spatial learning compared to the first. On the other hand, in novel object recognition testing, both genotypes were equally affected by the negative influence of social isolation. More importantly, however, we were also able to uncover a GxE interaction regarding the beneficial long-term effects of ENR. Whereas in rats previously housed under standard conditions, *Cacna1c* haploinsufficient animals performed worse than littermate controls during spatial learning, these differences between WT and HET were not seen in the ENR condition, and neither were the deficits observed in STD-housed HET males during the RAM reversal phase.

So far, no other study has employed social isolation as a stress factor in *Cacna1c* rodent models. As different types of stress are known to differentially affect brain and behavior (Burrows & Hannan, 2016), it is fair to assume that the generalizability of the negative effects of ISO observed in Study IV may be limited. At the same time, our findings are broadly consistent with evidence for GxE interactions in relevant *Cacna1c* mouse models. In two studies, the depletion of *Cacna1c* from the

NAcc (Terrillion et al., 2017b), as well as from forebrain glutamatergic neurons during early development (Dedic et al., 2018) led to an increased susceptibility to social defeat stress, whereas enhanced stress resilience was evident in mice that were virally targeted for knockout during adulthood. In this latter experiment, however, resilience was not evident at the level of cognitive functioning, with mice exposed to stress displaying impaired memory in the spatial object recognition test, irrespective of genotype (Dedic et al., 2018). Interestingly, when subjected to CUS in another GxE interaction study, initial deficits seen in mice of both genotypes in a spontaneous alteration WM task turned into resilience later in life for HET *Cacna1c* mice (Bavley et al., 2017). By contrast, the animals in Study III and IV did not display prominent alterations in working memory function, neither due to *Cacna1c* genotype, nor in terms of the effects of a potential GxE interaction.

The findings from Study IV also resonate well with clinical GxE studies. The human cohort investigated by Dedic and colleagues revealed an association between two intronic *CACNA1C* risk alleles and adverse life events with an altered risk for MDD, leading the authors to believe that MDD may only be predicted in individuals with a history of trauma (Dedic et al., 2018). In another study, MDD risk was investigated in *CACNA1C* risk allele carriers and healthy controls who had previously been exposed to TLEs. Both *CACNA1C* and TLEs were individually associated with MDD risk, yet the minor allele was found to exacerbate the risk conferred by TLEs (Zhao et al., 2019). Still more evidence pointing in this direction was obtained in a very recent study, finding that A-allele carriers of a different intronic *CACNA1C* variant were more likely to develop BPD when exposed to childhood trauma than non-risk allele carriers, indicative of a differential response to stress due to *CACNA1C* genotype (Bastos et al., 2020).

Especially the discoveries by Dedic and colleagues suggest a time-dependent gene-dosage link of potential interactions with ISO, as a complete knockout of *Cacna1c* in forebrain neurons during adulthood induced resilience to chronic stress in mice, which was not observed after embryonic deletion, and only partially in animals heterozygous for the gene (Bavley et al., 2017; Dedic et al., 2018). The importance of the forebrain in mediating GxE is further corroborated by a study conducted by Krautheim and colleagues who discovered higher schizotypal traits in individuals with a combined risk of SNP rs1006737 and childhood trauma, as well as deviant activity in the ventral ACC during outgroup emotion processing (Krautheim et al., 2018). The ACC and PFC seem likely candidate structures for the convergence of these interaction effects, as both adverse environmental influences (Baarendse et al., 2013; Erk et al., 2010; Frodl et al., 2010; Meyer-Lindenberg & Tost, 2012) and *CACNA1C* (Backes et al., 2014; Bigos et al., 2010; Erk et al., 2014a; Krug et al., 2010; Paulus et al., 2014) are known to influence prefrontal brain structures. In this context, it is possible that the effects of ISO on experimental groups with genetic risk may also be mediated via prefrontal BDNF, as early-life stress is connected to a

reduction of BDNF expression in adulthood (Roth & Sweatt, 2011), an alteration which has also been linked to *Cacna1c* (Kabir et al., 2017c).

To my knowledge, Study IV is the first to investigate the interaction of the *Cacna1c* risk gene with positive environmental circumstances in rats. However, our observations find support in other studies on rodents showing that environmental ENR can ameliorate behavioral outcomes with relevance to mental disorders. For instance, ENR can reverse deficits in animal models of autism (Schneider et al., 2006) and those induced by adverse environmental influences like prenatal stress (Laviola et al., 2004) or social isolation (Biggio et al., 2019). ENR has also been associated with positive effects in paradigms relevant to anxiety and sensorimotor gating deficits in psychotic disorders, such as exploratory behavior in the open field and prepulse inhibition (Joshi et al., 2017). Apart from neuropsychiatric disorders, ENR furthermore ameliorates learning and memory deficits in mouse models of Alzheimer's and Huntington's disease, as well as Down syndrome, and can enhance functional recovery of cognitive skills after a stroke (Nithianantharajah & Hannan, 2006).

It is conceivable that, just as the main effects discussed previously, the interaction of genotype and ENR on learning abilities in Study IV may rely on hippocampus-specific mechanisms that both environmental ENR and the *Cacna1c* gene are linked to. Substantiating evidence for this theory comes from experiments showing ENR can help mediate spatial learning deficits caused by hippocampal lesions in rats (Schrijver et al., 2004) and that deficits in mice with NMDA ablation in the CA1 region of the HC can be prevented by ENR (Rampon et al., 2000). Long-term potentiation, for example, is highly dependent on calcium influx (Woodside et al., 2004), and some forms of LTP specifically rely on proper functioning of voltage-gated calcium channels (Bauer et al., 2002) and are selectively involved in long-term memory formation. In relevant mouse models, an ablation of *Cacna1c* has already been implicated in the disturbance of late-phase LTP and associated spatial learning deficits in the MWM and the water cross maze (Dedic et al., 2018; Moosmang et al., 2005a). Environmental ENR, on the other hand, is known to promote LTP in the hippocampus (Artola et al., 2006; Cui et al., 2006; Duffy et al., 2001), as discussed at length in previous sections. Seeing that the synaptic plasticity of neurons in the rat dentate gyrus is selectively affected by ENR (Eckert & Abraham, 2010), long-term potentiation could very well represent a key mechanism where the effects of *Cacna1c* expression levels and an enriched post-weaning environment converge.

On top of that and as illustrated before, positive effects of ENR on neurogenesis are among the most replicated findings regarding research on environmental ENR (van Praag et al., 2000). For instance, ENR-induced improvements in learning performance demonstrably correlate heavily with adult hippocampal neurogenesis (Kempermann et al., 1997), and in terms of rescue effects, even the

cognitive decline in aged mice, which is associated with decreased neurogenesis in the DG (Kang et al., 2016; Kuhn et al., 1996) has been shown to be preventable via ENR (Bennett et al., 2006). As *Cacna1c* has been shown to influence neurogenesis in various experiments, it stands to reason that neurogenesis is altered in hippocampus of the HET rats in Study IV yet growing up in an enriched environment boosts neurogenesis and helps them perform on par with WT siblings. To highlight but a few of the neurogenesis-associated alterations in *Cacna1c* studies, a reduced number of immature neurons was found in the HC of forebrain- and neuron specific knockout mice (Lee et al., 2016; Temme et al., 2016), and the former study furthermore observed an increased rate of cell death in subject animals. By contrast, a study by Moon and colleagues could not corroborate the finding of fewer immature neurons in animals with *Cacna1c* ablation, but discovered significantly diminished cell proliferation rates in male HET rodents (Moon et al., 2018). Then again, recent experiments from our lab show that neither cell proliferation, nor rates of neuronal survival are markedly altered in female *Cacna1c* HET rats (Redecker et al., 2020), and therefore appear to refute the notion of neurogenesis being the main pathway of the interaction between ENR and *Cacna1c* haploinsufficiency observed in Study IV. Despite these negative findings, however, it is possible that in male rats as used for Study IV, differential findings may be obtained, since evidence is mounting that gonadal hormones affect adult hippocampal neurogenesis in a sex-dependent manner (Shohayeb et al., 2018). On top of this, the mechanism by which *Cacna1c* alters the neuronal life cycle in haploinsufficient rats may be more complex. For instance, it has been postulated that rather than affecting cell apoptosis and proliferation itself, a deletion of *Cacna1c* expression may specifically cause changes within the type-1 cells it is expressed in (Marschallinger et al., 2015; Temme et al., 2016; Völkening et al., 2017). The exact way in which ENR affects neurogenesis in the HC is furthermore thought to differ between rats and mice (Meshi et al., 2006), e.g. by rats displaying a larger rate of neurogenesis in general (Ellenbroek & Youn, 2016).

In a similar vein, the ENR-associated neurotrophic factor BDNF is also affected by *CACNA1C* (Sykes et al., 2019). LTCCs are known to mediate BDNF production in hippocampal excitatory neurons, and the loss of function in calcium channels has been thought to dysregulate BDNF transcription (Kabir et al., 2017c). BDNF is not only involved in mechanisms of neurogenesis (Scharfman et al., 2005) but also associated with early- and late-phase LTP in the hippocampus (Savitz et al., 2006). Hence, it is entirely possible that rescue effects in male HET *Cacna1c* rats during spatial and reversal learning are mediated via elevation of BDNF though environmental ENR (Ickes et al., 2000; Mosaferi et al., 2015). Interestingly, on top of cognitive abilities like reversal learning (Sakata et al., 2013), BDNF is also implicated in social behavior. BDNF-deficient mice show altered interactions with familiar conspecifics under anesthesia (Ito et al., 2019) and rodents exposed to social defeat stress exhibit altered BDNF

expression (Miczek et al., 2011). Most importantly, however, BDNF has been linked to social reward reduction as observed in MDD. Specifically, a role has been postulated for BDNF signaling between VTA and NAcc in mediating the susceptibility for social anhedonia (Kupferberg et al., 2016). An elevation of BDNF via ENR may therefore not only alleviate *Cacna1c*-associated cognitive deficits, but also the social anhedonia-like phenotype observed in the animals of Study I and II, which merits further investigation.

There also exists convincing evidence that alterations in mitochondrial functioning are associated with changes in the synaptic plasticity underlying cognition, as well as with neuropsychiatric disorders in general (Levy et al., 2003; Manji et al., 2012). Concerning calcium channels, mitochondrial dysfunction is known to have a negative effect on calcium regulation (Berk et al., 2011), and *Cacna1c* depletion has recently been linked to increased mitochondrial resilience to oxidative stress in a cell culture approach (Michels et al., 2018). In an effort to further investigate the underlying mechanisms of our observed GxE effect on behavioral outcomes in the RAM, we examined brain mitochondrial bioenergetics in PFC and HC tissue of *Cacna1c* WT and HET rats exposed to four weeks of post-weaning social isolation, standard housing or social and physical ENR (Michels et al., 2019). Even though mitochondrial function has been associated with *Cacna1c* and hypothesized to influence the brain's ability to adapt to different environmental stimuli, however, we did not find an effect of genotype on mitochondrial performance in either male or female rats, and no indication for a GxE interaction in terms of bioenergetics, membrane potential, reactive oxygen species production, or respiratory chain complex protein levels (Michels et al., 2019).

Relevance of the frontolimbic system

Genetic factors are known to modulate the effect of early life events on cognition (Short & Baram, 2019), yet the details of these processes, especially regarding *Cacna1c*, still need to be further elucidated. What becomes abundantly clear, however, is that the PFC-hippocampus-amygdala system appears to be highly relevant for the influence of *Cacna1c*, positive and negative environmental influences as well as their interaction on intermediate cognitive phenotypes in psychiatric disorders.

The PFC is known to regulate limbic brain regions in the sense of a top-down control, and in addition to the functional alterations of HC and PFC already described, dysregulation of this connectivity between the frontal lobe and the limbic system can result in distinctly impaired emotional regulation, attention, and cognition (Miller, 2000). Indeed, frontolimbic connectivity has been found to be altered in several mental disorders associated with social and cognitive dysfunction (Kahn et al., 2015; Richardson & Adams, 2018; Schafer & Schiller, 2018) and has been connected to *CACNA1C* risk variants in various studies (Dima et al., 2013; Paulus et al., 2014; Radua et al., 2013; Wang et al., 2011). At the

same time, environmental ENR is known to improve the connectivity between the hippocampus and neocortex (van Praag et al., 2000), highlighting an additional potential mechanism for the GxE interactions observed in Study IV.

Concerning learning and memory, frontolimbic connectivity is strongly linked to spatial and reversal learning (Pooters et al., 2015; Powell et al., 2015), as well as general RAM performance (Spieker et al., 2012; Young et al., 2009). However, the PFC is not only involved in working memory and cognitive control. Likewise, the HC does not only affect context processing and complex learning, and the amygdala does not merely code for stimulus salience. Next to the brain's reward system discussed earlier, all of these structures are also involved in social behavior. The PFC, for instance, is considered to be part of the "social brain" that mediates social behavior in humans (Porcelli et al., 2019) and rodents (Kabir et al., 2016). The ventral part of the PFC, in particular, is associated with facial affect processing (Dima et al., 2013) and social affiliation (Porcelli et al., 2019), and both ventral and dorsal PFC are involved in social cognition (Grady & Keightley, 2002). In the HC, increases in serotonergic signaling have been noted after social interactions (Felix-Ortiz & Tye, 2014) and abnormal activity is correlated with social impairment (Schafer & Schiller, 2018). The HC furthermore organizes social hierarchies and affiliative behaviors (Machado & Bachevalier, 2006). Interestingly, the size of an individual's social network can be predicted by their hippocampal-dependent memory, and processes within the HC, like adult hippocampal neurogenesis, have been repeatedly linked to social behaviors (Schafer & Schiller, 2018). Hence, it seems entirely possible and even highly probable that the *Cacna1c*- and housing-associated behavioral changes observed in Study I-IV are primarily mediated by the frontolimbic system, as also concluded by a recent review (Kabir et al., 2016).

On the whole, the behavioral results from Study IV indicate that rats harboring a constitutive haploinsufficiency for the *Cacna1c* gene are generally sensitive towards the influence of both positive and negative environmental factors on cognitive outcomes, and above all support the notion that the exposure to beneficial environmental circumstances during critical time windows may mitigate the risk for and detrimental course of learning and memory deficits in individuals with genetic risk conferred by *Cacna1c*. In this context, the frontolimbic system appears to be of relevance not only to the cognitive effects observed in Study III and IV, but also to the more prominent alterations in social behavior discussed further above.

Methodological considerations

Animal models of disorder-relevant phenotypes must demonstrate a certain degree of validity in the genetic manipulations, environmental influences and behavioral assays applied to be able to provide translational value for clinical application (Burrows & Hannan, 2016; Turner & Burne, 2013). Some

aspects of validity are of course being made somewhat redundant if one assumes that the symptoms of neuropsychiatric disorders overlap, and that current diagnostic categories are partly outdated. Nevertheless, the methods employed in Study I-IV will be discussed in the following paragraphs in terms of reliability and particularly face and construct validity, in the context of viable alternatives and with regard to potential considerations to be factored in when interpreting the results.

Genetic model

Species

For the subject of this dissertation, we employed a rat model with constitutive *Cacna1c* haploinsufficiency. By contrast, most of the existing literature focuses on genetically altered mice and has yielded more prominent behavioral alterations. Evidence suggests that this may be due to cellular and molecular differences between the two species that reflect on their behavior (Young et al., 2009). As an example, ENR may have differential effects on the HC and associated neurogenesis rates in rats and mice (Ellenbroek & Youn, 2016; Meshi et al., 2006), and in turn, the involvement of the hippocampus regarding performance in spatial paradigms apparently differs for the two species, as discovered in lesion studies. In mice, for instance, a 50% reduction of the dorsal hippocampus is sufficient to impair MWM abilities, whereas in rats, 80% are needed to cause significantly learning deficits (Silva et al., 1998). Naturally, our results would have been easier to compare with previous studies had we used mice. While both rats and mice are valid approaches to translational research (Morellini, 2013), however, rats offer certain advantages. There is a great deal of variability in mice, and several behavioral results in *Cacna1c* studies have been found not to be generalizable across genetic backgrounds (Sittig et al., 2016) and sometimes even across labs using the same mouse strain (Turner & Burne, 2013). On top of that, rats are known to perform more stably in cognitive paradigms (Ellenbroek & Youn, 2016; Young et al., 2009) and offer a wider spectrum of social behaviors, particularly in regard to play behavior and their employ of USV (Ferretti & Papaleo, 2019). Due to the fact that they start to play and communicate with conspecifics at a very young age, they also provide the means to investigate developmental social behavior in addition to interactions in adulthood (Berg et al., 2018). Changes in social environment, such as due to ISO, are also more effective in rats as the more social species of the two (Lapiz et al., 2003). In our opinion, rats provide greater translational value in the study of cognition and social behavior (Wöhr & Scattoni, 2013) and additionally enable the cross-species validation of mouse findings in *Cacna1c* research.

Knockout specificity

For the investigation of social communication, play behavior, spatial learning, cognitive flexibility and object recognition, we used a constitutive HET model, but there is great variation in genetic *Cacna1c*

rodent models in terms of region-specificity and knockout timing (see Table 4). Where we used constitutive HETs, other studies deleted *Cacna1c* in task-relevant regions, such as the forebrain, the CNS, or the hippocampus. The more prominent cognitive impairments discovered in those studies could be due to the fact that a 50% reduction of *Cacna1c* in the brain may not be specific enough for the manifestation of strong cognitive impairments, compared to a complete conditional ablation, or that expression patterns in specific brain areas play a key role. There are indications that in humans, too, *CACNA1C* is expressed in a tissue-specific manner (Kabir et al., 2017c) and that these expression levels are influenced by SNP risk variants (Bigos et al., 2010; Cosgrove et al., 2017; Gershon et al., 2014; Roussos et al., 2014). In rats, *Cacna1c* expression is highest in the DG of the hippocampus and the medial PFC, and lowest in the cerebellum (Sykes et al., 2018). Certainly, the investigation of specific brain regions is important to distinguish tissue- and task-specific effects of *Cacna1c*, and some tasks are known to depend heavily on certain regions, such as in hippocampus-dependent learning (Pooters et al., 2015) and in the dependence of playful interactions on specific brain areas (Pellis & Pellis, 2009). Then again, the constitutive HET model is considered to be better suited for translation to clinical studies than a full knockout, as a total absence of calcium channel function in humans is considered to be unlikely (Dao et al., 2010). Plus, in contrast to the more commonly used homozygous models which often elicit pronounced phenotypes but have weak validity with respect to affective disorders in humans, haploinsufficient animals offer the possibility of evoking the amelioration or exacerbation of an initially mild phenotype via environmental influences, such as physical and social ENR or social isolation (Kircher et al., 2018). Ideally, of course, one would contrast WT, HET and homozygous constitutive knockouts, but unfortunately, the latter option is not viable, as $Ca_v1.2$ null mice die of heart failure in utero (Seisenberger et al., 2000). Due to this embryonically lethality, no homozygous rat model was created.

Other studies have furthermore highlighted the importance of knockout timing by finding differential results in cognitive and social measures when inactivating *Cacna1c* in adulthood, as compared to during development. Indeed, $Ca_v1.2$ appears to differentially contribute to the acquisition and maintenance of cognitive abilities across the lifespan. For example, Dedic and colleagues observed a drastic cognitive impairment in mice harboring a developmental knockout of *Cacna1c*, yet an almost opposite phenotype if *Cacna1c* was targeted during adulthood, where animals demonstrated improved cognitive flexibility, strengthened synaptic plasticity and resilience towards chronic stress (Dedic et al., 2018). Regarding human risk allele carriers, it is yet unclear whether the changes in brain anatomy observed in clinical *CACNA1C* studies arise during early development, during adulthood, or both (Bhat et al., 2012). However, compared to animals with *Cacna1c* knockout in adulthood, developmental knockouts appear to be more sensitive to environmental influence (Dedic et al., 2018),

especially if these coincide with critical periods of development for essential brain structures like the HC (Bigos et al., 2010; Lapiz et al., 2003), which is especially relevant for the findings from Study IV. The possibility needs to be taken into account, though, that in constitutive developmental HET models, compensatory processes may be at play that invalidate the knockout effect in certain paradigms (Kabir et al., 2016; Langwieser et al., 2010; Salama & London, 2007; Terrillion et al., 2017a) and that these mechanisms may also be sex-dependent (Lenz et al., 2012). It has been suggested that they can be circumvented if – depending on the knockout method – calcium channels are repeatedly blocked via viral vectors in adulthood (Kabir et al., 2016) or if the initial knockout is introduced at a later point in time during postnatal development (Lee et al., 2016).

In the rats used for Studies I-IV, we furthermore induced a reduction in *Cacna1c* expression, similar to some of the *Cacna1c* studies in mice (Bavley et al., 2017; Dedic et al., 2018; Moosmang et al., 2005a; Temme et al., 2016) and as verified via Western blot in Study I. However, there are other studies with findings of cognitive and social deficits on a behavioral level that generated a *Cacna1c* knock-in model (Bader et al., 2011; Kabitzke et al., 2018). Unfortunately, the exact influence of *CACNA1C* SNP risk variants on mRNA expression and channel function is not clarified yet. Most SNPs implicated in GWAS are located in a large intronic region of the gene coding for CACNA1C and are therefore thought to affect gene expression, rather than alter channel functionality (Moon et al., 2018; Roussos et al., 2014). In this context, studies investigating the effects of risk variants on mRNA expression in cell cultures and postmortem tissue have yielded differential results, and both increased (Bigos et al., 2010; Yoshimizu et al., 2015) and decreased (Gershon et al., 2014; Roussos et al., 2014) levels of *Cacna1c* mRNA have been linked to psychiatric disorders. Then again, an increase in mRNA levels does not necessarily lead to an increase in protein expression (Bavley et al., 2017). Although the disruption of *Cacna1c* mRNA is more likely to be associated with loss of function in calcium channels (Heyes et al., 2015), some mutations are decidedly connected to an increase of Ca^{2+} currents and reduced inactivation (Splawski et al., 2004). Despite these discrepancies, it is conceivable that dysregulation of calcium channels in either direction may cause a disruption of neuronal pathways and consequently alter behavioral outcomes (Heyes et al., 2015; Kabir et al., 2017c) as intracellular calcium is essential for a variety of cellular signaling processes (Bhat et al., 2012; Casamassima et al., 2010; Simms & Zamponi, 2014; Striessnig et al., 2014) and both loss and gain of function models are valuable and necessary to pin down why exactly *CACNA1C* is so heavily implicated in neuropsychiatric disorders (Kabir et al., 2016). Some authors have hypothesized that a haploinsufficient or region-specific full knockout of *Cacna1c* may even exert a protective influence on behavior (Dao et al., 2010; Zanos et al., 2015), although it seems counterintuitive that the WT variant represents the “risky” version of the gene. The effect, however, may be domain-specific, such as on depressive-like behavior (Dao et al., 2010; Kabir et al.,

2017b) and taking into account the effects observed in Study IV, the HET genotype may open up the organism not only to adverse, but also to beneficial experiences, in the sense of a differential sensitivity for environmental influences (Assary et al., 2018).

Environmental manipulation

In humans, the investigation and comparison of environmental factors is remarkably challenging because of the plethora of social and physical stimuli in an individual's environment, which are all different in nature and hard to quantify (van der Auwera et al., 2018). In Study IV, we used four weeks of either post-weaning social isolation or physical and social ENR with several weeks subsequently spent in standard housing until testing commenced.

Housing conditions

As is common for social isolation protocols, our rats still had access to auditory, visual and olfactory stimuli from conspecifics but no means to directly interact (Fone & Porkess, 2008). As such, ISO constitutes an extreme version of sensory, cognitive and motor deprivation (Burrows et al., 2011) but provides certain advantages over other potential stressors including CUS, social defeat and prenatal stress (Sandi & Haller, 2015). Adverse experiences during early life have a big impact on cognitive abilities, as shown in humans, and the relationship is known to be complex (Short & Baram, 2019). However, ISO constitutes a non-pharmacological intervention that consistently produces long-term changes in behavior, brain anatomy and function with relevance to neuropsychiatric disorders (Fone & Porkess, 2008) and is considered to be a convenient and ethologically appropriate manipulation for environmental risks, as for example in SCZ and MDD (Lapiz et al., 2003).

Environmental ENR protocols, on the other hand, vary greatly across labs. There are differences regarding cage size and composition, social and inanimate stimulation, inclusion of a running wheel, bedding material and the exchange of objects (Bennett et al., 2006; Nithianantharajah & Hannan, 2006), but it usually comes down to an increased range of opportunities for sensory, cognitive and physical stimulation in a larger cage filled with a variety of objects to climb on, hide in, play with and explore (Burrows et al., 2011). As of today, there is no consensus as to which ENR protocol yields ideal results, yet the details of ENR may be somewhat irrelevant in terms of effects on individual variability or reliable replication. Indeed, while post-weaning ENR cannot mirror the plethora of experiences and interactions a human child makes during adolescence, it is known to produce positive behavioral and neurobiological changes across labs, species and individual strains (Nithianantharajah & Hannan, 2006), and has been consistently associated with enhanced learning and memory (Bruel-Jungerman et al., 2005; Leggio et al., 2005; Simpson & Kelly, 2011). We did not include a running wheel in the cage setup because physical activity itself is known to promote spatial learning, BDNF secretion and cell

proliferation in the DG, independent of environmental ENR (Kempermann et al., 1997; Leggio et al., 2005; van Praag et al., 2000). However, ENR can enhance these measures without the incorporation of a running wheel in the cage setup, as demonstrated in a previous study conducted in our lab (Brenes et al., 2016). At the same time, it is important to frequently exchange objects within the cage, as ENR rats habituate quickly to novelty (Schrijver et al., 2002) and repeated substitutions increase the complexity of sensory exposure (Burrows et al., 2011). We also regularly hid treats throughout the cage to encourage foraging and exploratory behavior (Simpson & Kelly, 2011). Interestingly, while both social and inanimate stimulation play an important role in behavioral and brain development (Schrijver et al., 2002), it is the physical component of ENR that appears to influence learning performance most prominently (Brenes et al., 2016; Schrijver et al., 2004; Toyoshima et al., 2018; Wang et al., 2017), whereas the social component alone cannot elicit the same positive effects on cognition as complex social and physical ENR (Burrows et al., 2011). In summary, the current general consensus is that social and physical ENR is the best translational model for the investigation of the protective and curative influence of positive life experiences in humans (Nithianantharajah & Hannan, 2006; van Praag et al., 2000).

In addition to cognitive outcomes, both ISO and ENR manipulations have proven effects on social behavior, creating the possibility to investigate GxE effects on social interaction and communication in *Cacna1c* animals under similar conditions as in Study IV. This comes as no surprise as isolated animals, specifically, lack experience in social interaction with conspecifics apart from mother and siblings (Burke et al., 2017a) and cannot participate in rough-and-tumble play, which is known to peak during this time (Panksepp, 1981). This can result in significant communication and interaction deficits, the extent of which depends on the duration of ISO and subsequent re-socialization (Hol et al., 1999; Seffer et al., 2015; Templer et al., 2018). In human children, early environmental enrichment has furthermore been suggested to lead to less antisocial behavior in adulthood (Leggio et al., 2005), although rodent studies indicate that the type of ENR is critical, as social and physical aspects can have differential effects on social behavior (Brenes et al., 2016).

Timing

Regarding the exact timing of environmental manipulation, there is evidence that differential results can be obtained if animals are exposed to ISO or ENR for an extended period (Hullinger et al., 2015) or at a different timepoint during development (Fischer et al., 2012). In Study IV, we were only able to detect a mild advantage of ENR over other housing conditions. Evidence for a stronger effect on behavior due to longer ENR is provided in a study by Hullinger, whose ENR rats were on par with STD animals in terms of object recognition abilities after one month of ENR but delivered an outstanding

performance after four months (Hullinger et al., 2015). However, we chose the four weeks following weaning on PND21 as this period is thought to mirror the time window of greatest vulnerability in humans. In most disorders, the first behavioral symptoms emerge during adolescence (Burrows et al., 2011), and in rodents, the post-weaning phase is known to be a critical time window for the shaping of brain (Lapiz et al., 2003; Short & Baram, 2019) and behavioral phenotypes by the environment (Sachser et al., 2018). By contrast, environmental manipulation pre-weaning can be confounded by maternal effects like licking and grooming (Nithianantharajah & Hannan, 2006).

Another point for discussion is the delay until testing. Other studies have yielded differential results in reversal learning abilities (Powell et al., 2015) or in the response to stress, depending on the time point of testing relative to the occurrence of environmental housing (Bavley et al., 2017). For all we know, more marked effects of ENR and ISO, as well as GxE might have been observed if testing had commenced sooner after the end of environmental manipulation. Subsequent to ISO and ENR, the animals from Study IV furthermore spent several weeks in conditions where they shared a cage with littermates harboring a different genotype, and with varying environmental experience. Hence, apart from a mere effect of time, the moderate phenotypes elicited by genetic and environmental manipulation as observed in Study IV might also be due to "inclusion" phenomena, in that the mixed housing muted the effects of genotype and environment. As inclusion effects have previously been found to normalize the influence of both genotype (Yang et al., 2011) and environment (Seffer et al., 2015), this possibility cannot be precluded from affecting the results. For example, ISO and STD animals are known to have opposing patterns of USV emission, yet ISO animal can adopt a more STD-like pattern of vocalizations once back in social housing (Panksepp & Burgdorf, 2000). Notably, rescue effects via re-socialization are not only apparent on the behavioral level but can also manifest in elevated cell proliferation and BDNF expression (Biggio et al., 2019). Then again, STD housing after ENR does not necessarily expunge or attenuate the effects of environment (Simpson & Kelly, 2011), as shown by one study where mice spent almost 10 weeks in STD housing after ENR and did not differ from those who were continuously enriched in terms of spatial learning performance or cell proliferations rates (Kempermann & Gage, 1999).

On the one hand, our reasoning for the long delay before cognitive assessment was of an organizational nature, as the animals described in Study IV also underwent other paradigms prior to NOR and RAM. However, the time difference to ENR and ISO also enabled us to show that a moderate influence of four weeks of environmental manipulation was still evident in adult rats housed in groups under standard conditions for several weeks after the exposure to social and physical environmental ENR had ended. In terms of cage composition, we furthermore believed that the benefits of housing littermates together would outweigh the risk of potential rescue effects. In any case, in our opinion a

mixed housing more accurately mirrors conditions in the "real world" where individuals with certain experiences or genetic risk might flock together more often than by chance, but are not confined to only interact with specific groups. Nevertheless, same-genotype and same-environment housing following environmental manipulation might provide valuable insights in future studies.

Assessment of cognition

Novel object recognition

For the investigation of recognition abilities, we used the novel object recognition paradigm, since recognition memory capitalizes on similar brain structures in humans and rodents (Young et al., 2009). As a subtype of declarative memory, it is often impaired in patients with HC damage (Wallace et al., 2015) or in disorders like SCZ and MDD (Dere et al., 2010). The NOR relies on the natural preference of rodents to explore unfamiliar stimuli (Ennaceur & Delacour, 1988), and thus provides the possibility of efficiently examining non-spatial object recognition abilities in just one test trial (Dere et al., 2007). Moreover, due to the spontaneous nature of the task, no aversive stimulus or reward is required (Bevins & Besheer, 2006; Wallace et al., 2015) which might increase stress for the animal or be differentially affected by genotype (Lancaster et al., 2014) or housing condition (Rose et al., 1986). This aspect also increases the comparability of NOR to recognition tasks in clinical studies which do not require extensive pre-training (Dix & Aggleton, 1999). In addition, the ITI can be increased in length to increase the difficulty for animals to recognize the unfamiliar object during the recognition trial. Indeed, variations in task difficulty might help to reveal more profound GxE interactions, as hinted at by several studies that observed a prominent effect of ENR depending on the ITI (Bruel-Jungerman et al., 2005; McLean et al., 2010; Melani et al., 2017) or additional distracting stimuli (Jones et al., 1991). Regarding *Cacna1c*, a differential effect on genotype may likewise be expected with increased task difficulty, as previously explained (Temme et al., 2016).

We habituated the animals to the arena 24 hours in advance and chose a test trial lasting five minutes in Study III and IV. While longer durations can be chosen to see if differences emerge later in the trial, one study investigating the most sensitive time period of the NOR test phase found that the clearest distinction is made by animals in the first two minutes of the trial, regardless of ITI (Dix & Aggleton, 1999). Naturally, though, objects need to have distinct features without encouraging interaction apart from exploration (Bevins & Besheer, 2006) and be counter-balanced in terms of choice of the novel item and location in the arena to avoid effects of a natural bias.

In general, the NOR is considered to adequately model visual learning and memory and to be well suited for the investigation of recognition memory deficits in translational research (Keeler & Robbins, 2011; Young et al., 2009). However, even though the reduction in pre-training requirements provides

easy implementation of the NOR, this fact can be disadvantageous in the sense that the paradigm of course lacks the possibility to assess differences in the ability to learn, which is one of the reasons why we employed an additional paradigm measuring cognition in the RAM.

Radial arm maze

Unlike rodent versions of paradigms employed for recognition memory, which are widely considered translationally valuable tests, the episodic memory equivalent in rodent spatial navigation has given rise to doubts about the comparability of human and rodent cognitive abilities in the past (Morellini, 2013). Nonetheless, by now the use of mazes that require allocentric navigation is considered an acceptable rodent equivalent, since performance is susceptible to ageing (Pooters et al., 2015) and largely dependent on the hippocampus, which is also thought to primarily underlie human episodic memory (Morellini, 2013).

Whereas the partially baited RAM was used for Study III and IV, several other experiments, especially those with regard to *Cacna1c*, have employed the Morris water maze as a spatial learning paradigm which is known to lead to quick learning, while the acquisition of the RAM appears to be more challenging (Vorhees & Williams, 2014). Considering that different results were obtained in *Cacna1c* animals depending on specific task demands, e.g. in classic and observational fear conditioning (Jeon et al., 2010), the construct validity of a paradigm is essential to pinpoint which type of learning is impaired, which is why performance in different types of mazes cannot simply be generalized (Hodges, 1996). Both the RAM and MWM are well-established paradigms to test spatial learning in rodents, yet there are several differences to be considered. For example, in contrast to the food-incentivized RAM, the MWM, just as the water cross maze (Dedic et al., 2018), is motivated by an escape to safety which may contribute to the steep learning curves in almost all rodent strains subjected to the task (Vorhees & Williams, 2014). However, while some modifications, like the water cross maze, aim to reduce the swim stress on rodents, the MWM can be easily influenced by basic motor function (Vorhees & Williams, 2014), although some of the *Cacna1c* mouse studies specifically controlled for thigmotaxis and exploratory behavior as potential confounding variables (Moosmang et al., 2005a). Performance on the MWM is furthermore thought to vary in a sex-dependent manner (Simpson & Kelly, 2011) and to differ for animals exposed to environmental housing conditions, as ENR rats demonstrate a significantly higher swim speed (Larsson et al., 2002).

Compared to other maze paradigms, the RAM is thought to mirror the environmental demands on rodent abilities more closely, as spatial navigation is highly relevant in the search for food in a rat's natural habitat. Just as in an organic foraging environment, rats need to switch between a "win-stay" and a "win-shift" strategy when sustenance is no longer available in a certain patch of ground,

providing the RAM with increased ethological validity (Young et al., 2009). As such, the test allows the investigation of learning between trials, as well as within trials and can assess spatial reference memory and spatial working memory simultaneously, whereas the spontaneous alteration in T- and Y-mazes concentrates on spatial working memory, and other tests, like the Morris water-maze, primarily examine reference memory (Hodges, 1996; Morellini, 2013).

In GxE research, especially, it is paramount to establish test protocols analogous to human tests (Burrows & Hannan, 2016). As SCZ patients are impaired in paradigms requiring allocentric navigation (Young et al., 2009), and specifically in a human virtual RAM task (Spieker et al., 2012), the RAM fulfills this criterion whereas the relevance of the MWM in clinical neuropsychological tests has been judged limited (Young et al., 2009). Then again, Koppe and colleagues argue that *Cacna1c* rats might employ different search strategies (Koppe et al., 2017) which are more easily assessed in the MWM (Hodges, 1996). The rate of success on the RAM can obviously be increased by the animal employing an adjacent arm strategy (Juraska et al., 1984), which we did not control for. However, at least regarding environmental effects, other studies controlled for search strategies by limiting the access to arms with doors and found beneficial effects of ENR on RAM performance, regardless of search strategy (Leggio et al., 2005). We did attempt to forestall egocentric navigation strategies by placing the rat on the maze facing a different direction with each trial. Also, and like in RAM testing, the navigation by proximal olfactory cues was prohibited by thorough cleaning of the maze with ascetic acid between trials.

In contrast to the MWM, the range of different choices to be made in the RAM is limited (Vorhees & Williams, 2014), although the paradigm is open to extensions. A stronger influence of *Cacna1c* genotype, for example, may be expected under more difficult conditions, such as in a limited cues version of the maze (Temme et al., 2016), an increase of potential errors due to a larger number of arms (Vorhees & Williams, 2014) or longer intervals preceding a probe trial. In fact, the inclusion of remote probe trials is highly recommended for future studies, as studies have shown that differential result patterns may emerge depending on probe trial timing (White et al., 2008).

In the end, there are no perfect paradigms uniquely appropriate for research on cognitive deficits in neuropsychiatric disorders. Each has their advantages and disadvantages depending on species (Morellini, 2013) and whether the focus is on the assessment of WM in addition to RM, or on the elimination of procedural search strategies as potential explanations for behavioral differences (Leggio et al., 2005). An exact replication of human paradigms in preclinical tests for rodents cannot be expected, as e.g. language is an integral part of human problem solving, such as during memory tests where participants are known to repeat information verbally in their mind (Young et al., 2009).

However, the validity and relevance to clinical studies can be improved if the same brain circuitry is involved in task completion (Keeler & Robbins, 2011). The MWM and RAM are known to be HC-dependent (Morellini, 2013; Woodside et al., 2004), but both intact HC and PFC are necessary for success on the RAM (Spieker et al., 2012), since reversal learning requires cognitive flexibility and executive function (Morellini, 2013). Finally, and as highlighted before, working memory deficits are highly prevalent in various disorders (Barch & Ceaser, 2012; Dere et al., 2010; Heck et al., 2014) and among the two paradigms, only the RAM is uniquely equipped to assess these alongside spatial reference memory (Turner & Burne, 2013).

Behavioral confounds

Several further behavioral constructs are related to and potentially influence cognition and social behavior, are altered in mental disorders and associated with *CACNA1C*. These include, but are not limited to, anxiety, emotional regulation, and general perception and locomotor abilities. If impaired in a genotype- or environment-specific fashion, these behavioral confounds may endanger the construct validity of the paradigms discussed so far.

Emotion & anxiety

Phenomena like a cognitive bias towards negative interpretations of ambiguous information in MDD (Millan et al., 2012) and the selective encoding and recall of memories depending on the current affective state (Dere et al., 2010) are just two examples of how emotional differences can potentially impact performance in cognitive paradigms. The current mood of an individual also impacts their desire for social contact, behavior during social interaction and receptiveness for social stimuli, which is highly relevant for Study I and II. On top of this, high levels of anxiety can modulate memory formation (Dere et al., 2010), as well as impact a rodent's behavior in tests that require spontaneous exploration, like the RAM and NOR (Hodges, 1996). Anxiolytic mediation, in turn, is known to increase social interactions (Kabir et al., 2017a).

Notably, emotional dysregulation, as well as elevated anxiety are two distinct intermediate phenotypes seen in most major neuropsychiatric disorders and both have been linked to *CACNAC1C* (Kabir et al., 2016; Nieratschker et al., 2015). In fact, in humans, *CACNA1C* risk variants have been associated with higher anxiety and depression scores (Erk et al., 2010; Roussos et al., 2011), altered brain activity during emotional processing (Bigos et al., 2010; Tesli et al., 2013), impaired facial emotion recognition (Nieratschker et al., 2015), and higher emotional lability (Strohmaier et al., 2013). While rodents do not exhibit specific moods per se, aberrations in emotional learning (Bader et al., 2011; Jeon et al., 2010; Kabir et al., 2017a; Temme & Murphy, 2017), as well as increased anxiety levels (Bader et al., 2011; Dao et al., 2010; Dedic et al., 2018; Kabir et al., 2017a; Lee et al., 2012) have been

noted in relevant *Cacna1c* mouse studies. Due to the high probability of confounding social and cognition-associated behavior, these *Cacna1c*-specific findings on emotional regulation and anxiety need to be considered in the translation from our findings to clinical research. For instance, the initial hypoactivity in the HET *Cacna1c* females of Study III, as well as the insistence on outdated behaviors in the face of novelty seen in HETs during reversal learning can potentially be interpreted as rather anxious behavior that was ameliorated by ENR in Study IV (Kang et al., 2016). Concerning the reported influence of ISO on heightened anxiety (Schrijver et al., 2002), however, we can report that this, at least, was not apparent in the display of exploratory behavior during NOR in Study IV and may thus not explain the prominent object recognition deficits observed in the animals reared in social isolation.

Perception

Olfactory abilities are highly relevant for daily rodent life and the basis for normal social interaction (Ferretti & Papaleo, 2019), which is why we controlled for this ability via the olfactory habituation and dishabituation paradigm in our *Cacna1c* haploinsufficient rat model. This test is used for verification of a rodent's capacity to distinguish between different social and non-social odors and remember familiar scents (Yang & Crawley, 2009). The *Cacna1c* HET rats used in Study I-IV did not display any impairments in this paradigm, which could potentially confound the social interaction findings from Study I and II or reduce the incentive value of sucrose pellets on the RAM as used in Study III and IV. This is consistent with a previous study in HET mice, where the time to find food in the hidden cookie test was not affected by *Cacna1c* genotype (Dao et al., 2010). Likewise, visual discrimination does not appear to be affected by *Cacna1c* (Sykes et al., 2019) which is noteworthy as intact visual perception enables spatial orientation on the RAM and may support novel object recognition (Ennaceur & Delacour, 1988). Also, environmental influences are known to influence the occipital cortex, which is highly relevant for visual abilities (Rose et al., 1986). As a third sense, intact auditory processing is a necessary prerequisite for the correct interpretation of behavior in the USV playback paradigm employed in Study I and II. However, immediate head orientation in response to the presentation of 50-kHz USV and 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, indicative of intact auditory perception in WT and *Cacna1c* HET animals.

Locomotion

For tasks that require the animal to move about on a platform or in an arena, such as for arm exploration on the RAM, reaching the object in NOR, social approach behavior in the USV playback paradigm or play behavior in general, intact locomotor abilities are an essential behavioral feature that can act as a potential confound on the results. Several *Cacna1c* studies have discovered altered

novelty- and socially-induced locomotion in the TS2-neo mouse, even though basal locomotor activity appears to be intact (Bader et al., 2011; Kabitzke et al., 2018). Then again, HET *Cacna1c* knockout mice displayed hypoactivity in the activity chamber, as well as in their home cage compared to control littermates (Bader et al., 2011). Additionally, decreased exploratory behavior and an attenuated locomotor response to amphetamine were observed in female animals with partial *Cacna1c* ablation (Dao et al., 2010). In haploinsufficient *Cacna1c* rats, similar findings of reduced locomotion in females were obtained in another playback study conducted in our lab (Wöhr et al., 2019), matching the initial hypoactivity observed in arm entries of the female HETs in Study III. By contrast, the time spent exploring two objects in the acquisition trial of the NOR was quite comparable between the two genotypes. While there were no further significant differences in locomotor activity, it needs to be mentioned here that on a mere descriptive level, HET rats explored the objects a little less than WT littermates during the NOR acquisition trials in Study III and IV, and male STD-housed HETs also appeared to enter fewer arms during spatial learning in both cohorts.

In this context, a comment on potential cardiac phenotypes in *Cacna1c* HET rats is required. In human subjects, the *CACNA1C* rs1006737 risk allele was hypothesized to induce changes in the cerebral blood flow (Ou et al., 2015), which seems plausible considering that $Ca_v1.2$ is the predominant calcium channel in the human heart (Striessnig et al., 2014) and constitutive *Cacna1c* deletion causes heart failure in mice (Seisenberger et al., 2000). Both increased and decreased *Cacna1c* activity is thought to predispose for cardiac disease (Striessnig et al., 2014) and HET $Ca_v1.2$ mice have been shown to develop cardiac hypertrophy when subjected to stress (Goonasekera et al., 2012), which is why special attention needs to be paid to potential behavioral effects due to cardiovascular changes. Then again, while a complete knockout caused a reduction of myocardial contractility and subsequently increased lethality in mice carrying a homozygous $Ca_v1.2$ deletion, the HET genotype was found to be notably less affected, with unchanged cardiac function (Rosati et al., 2011). Unpublished data from the haploinsufficient animals used in our lab reveals, however, that although they showed no differences in heart rate, hypo- or hypertrophy, HET *Cacna1c* rats did display a decrease in blood pressure (Fender et al., 2019) which might explain the decreased locomotor activity.

Although we do not believe that this significantly altered learning performance or social approach behavior, these sex-specific trends towards reduced locomotion in rodents heterozygous for the *Cacna1c* gene call for further investigation to ensure the validity of the obtained results.

Sex differences

Most *Cacna1c* research so far has focused on males, which is astounding, as the National Institute of Health introduced a major policy change several years ago on the balance of both sexes in preclinical

research (McCullough et al., 2014). On the other hand, there may be an increased behavioral variability due to the estrous cycle and associated hormonal changes in female rodents (Meziane et al., 2007; Mora et al., 1996; Yoest et al., 2019) which was one of the reasons why we chose male rats for the GxE investigation in Study IV. Nevertheless, the practice of examining mostly males neglects the sex-dependent findings regarding neuropsychiatric disorders in humans. Not only do the prevalence rates and disorder course of MDD (Kendler et al., 2005; Mullins & Lewis, 2017) and SCZ, as well ASD (Chaste & Leboyer, 2012; DiLalla et al., 2017) differ depending on biological sex, but *CACNA1C*-specific findings also point to a differential effect of intronic risk variants in women, as compared to men. For instance, the influence of rs1006737 on diagnosis, associated personality traits, and relevant resilience factors was reported to be more prominent in women by several authors (Dao et al., 2010; Heilbronner et al., 2015; Strohmaier et al., 2013; Witt et al., 2014). Additionally, in a very recent study, another risk SNP was associated with increased hostility, less emotional intelligence and higher harm avoidance tendencies in healthy females, but not in male carriers of the minor allele (Takeuchi et al., 2018). Regarding behavioral domains in rodents, we established sex differences especially in the social behavior of female *Cacna1c* rats in Study II, yet also trends towards differential efficiency and cognitive flexibility in Study III. In terms of the latter, potential explanations may be sought in critical periods of brain development of relevant cognitive circuits in the two sexes (Lenz et al., 2012; Short & Baram, 2019), as described in previous sections. We did not include female animals in the GxE study due to the resource-intensive nature of RAM testing. However, it is paramount to investigate further into potential environmental and GxE effects in *Cacna1c* females, too, as observations may shed light on the varying responses of men and women to stress (Hasler et al., 2004), just as male and female rodents respond differently to the same environmental challenge (Burrows et al., 2011; Simpson & Kelly, 2011).

Translational value & future perspectives

The four studies on social behavior and cognitive abilities in rats presented here add to the growing body of literature suggesting that the *Cacna1c* gene plays a significant role in the alteration of behavioral phenotypes associated with neuropsychiatric disorders. In clinical studies, corresponding findings were obtained in aberrant social behavior as well as learning and memory deficits displayed by human *CACNA1C* risk allele carriers. As both social and cognitive dysfunction are highly prevalent and strongly debilitating for patients of MDD, BPD, SCZ and ASD, our results aim to elucidate which cognitive and social domains exactly are affected by genotype or adverse experiences and which aspects may be amenable to positive environmental influences.

Regarding spatial learning, we observed a slight retardation but good long-term learning on the RAM in HET *Cacna1c* rats under STD conditions, with deficits being absent if animals were reared in enriched

housing. Apart from other *Cacna1c* rodent studies which have already been discussed at length, our pattern of results closely resembles findings obtained in humans that show *CACNA1C* minor allele carriers can generally learn, just not as well as those unencumbered by genetic risk, as displayed by an initially poorer performance in the verbal learning and memory test (Dietsche et al., 2014). As it has been shown that learning abilities in rats have an episodic memory-like quality (Panoz-Brown et al., 2016), Study III and IV suggest that *Cacna1c* does not impede the ability to acquire and retain knowledge per se, but rather modifies the speed and depth of learning, and that additional factors must be at play in the more profound cognitive deficits observed in other studies on human risk allele carriers (Backes et al., 2014; Hori et al., 2012; Krug et al., 2010).

In terms of working memory, we observed no prominent impact of either genotype or environment which matches the generally mixed findings in clinical studies. Like us, other authors detected no association between *CACNA1C* and WM (Paulus et al., 2014; Rolstad et al., 2016; Takeuchi et al., 2018) although this relationship appears to depend on diagnosis (Arts et al., 2013; Cosgrove et al., 2017; Hori et al., 2012; Soeiro-de-Souza et al., 2013). It is not entirely clear whether the increases and decreases in working memory capacity (Heyes et al., 2015) may be linked to the increases and decreases of *CACNA1C* mRNA seen in postmortem brain tissue (Bigos et al., 2010; Gershon et al., 2014) or if other factors like negative experiences in critical time windows of development influence the results. In fact, the initial inferiority of ISO rats in Study IV supports the notion that adverse life events can exacerbate cognitive deficits and may result in impaired WM, as assessed in BPD patients exposed to childhood trauma (Bücker et al., 2013; Schenkel et al., 2005).

The biggest alteration in HET animals was discovered in an initial impairment of cognitive flexibility, which did not hinder reversal learning in general and was rescued by four weeks of ENR. By contrast, there is little evidence that implicates *CACNA1C* in executive function in healthy subjects, yet stronger associations in BPD patients (Chelune & Baer, 1986; Lin et al., 2017; Rolstad et al., 2016; Soeiro-de-Souza et al., 2013). Notably, the ascribed inferiority of reversal learning abilities in human risk allele carriers appears to be driven by trials in closer temporal proximity to contingency reversal (Sykes et al., 2019) which corroborates the pattern of behavior observed in Study III. Reversal learning and cognitive flexibility tests are often used to mirror impulsivity, compulsive perseverance and self-regulation abilities, and especially in today's fast changing world, the inability to adapt to new circumstances poses a substantial problem for affected individuals. Together with findings from clinical studies, our results indicate that *Cacna1c* plays a role in initial adaptation difficulties but does not prevent long-term adjustment to new circumstances. Behavioral outcomes may be better for individuals with a good social support system in contrast to those never exposed to protective influences, as indicated by Study IV and the significant associations of the *CACNA1C* risk allele with

executive function in patients, but not healthy controls (Lin et al., 2017; Rolstad et al., 2016; Soeiro-de-Souza et al., 2013).

Combined with relevant studies in human risk allele carriers (Dietsche et al., 2014), the NOR results from Study III and IV give no indication to believe that potential deficits in recognition memory are due to variation in *CACNA1C* alone, yet it is conceivable that the strong impairments seen under ISO housing denote the relevance of a history of trauma, as also suggested by findings of impaired novel object recognition in a rat model for post-traumatic stress disorder (Eagle et al., 2013). Then again, *CACNA1C* is by far not the only gene implicated by GWAS (Visscher et al., 2017), and gene by gene interactions are a possible additional influence factor to be considered in future GxE studies (Kaufman et al., 2006).

In general, cognitive impairments are heavily linked to disorder course and relapse rate (Hammar & Ardal, 2009) and an important predictor of functional outcomes and recovery, independent of depressive of psychotic symptoms (Green et al., 2000; Millan et al., 2012). In this context, the GxE results from Study IV are of particular relevance, as they show that even *Cacna1c* HET rats who show impaired cognitive flexibility and learning retardation under standard conditions can benefit from positive experiences. In humans, these observations may inform treatment and intervention methods that can alleviate cognitive deficits and consequently improve quality of life (Millan et al., 2012).

Next to cognitive dysfunction, social interaction and communication deficits often cause social withdrawal. This, in turn, can lead to greater social deficits as the adaptation and conformation to social customs is not practiced, consequently reducing the ability of individuals to participate in society. In Study I and II, male and female HET *Cacna1c* rats displayed altered rough-and-tumble play and impairments of pro-social communication in both sender and receiver of USV. As $Ca_v1.2$ has been heavily implicated in both the amygdala and dopamine-signaling in the brain's reward circuit, these observations indicate potentially deficient salience coding for social stimuli and reduced incentive value for pro-social 50-kHz USV, as well as direct social interaction with a conspecific in animals with partial *Cacna1c* ablation. Similar deficits in facial affect processing (Dima et al., 2013; Nieratschker et al., 2015; Soeiro-de-Souza et al., 2013), reward responsiveness (Lancaster et al., 2014), and social behavior (Roussos et al., 2011; Takeuchi et al., 2018) have been observed in human subjects carrying the minor variant of *CACNA1C* risk alleles. As relevant brain structures like the amygdala and NAcc display significant alterations in their volume (Frazier et al., 2014; Lancaster et al., 2016; Perrier et al., 2011) as well as their activity during reward (Wessa et al., 2010) and social stimulus processing in human risk allele carriers (Dima et al., 2013; Jogia et al., 2011; Sumner et al., 2015; Tesli et al., 2013), these interpretations find support in clinical studies on neurobiological measures. Together, the results

from Study I and II suggest that *Cacna1c* appears to play a role either in the incorrect interpretation of social cues or in the reduced rewarding quality of social interactions, both of which may contribute to aberrant communication and interaction behavior in social situations and result in a phenotype of social anhedonia.

Moreover, environmental factors are known to contribute to behavioral alterations in social interaction and social cue processing, such as the ability to recognize facial emotions (Aas et al., 2016; Holmes, 2019). It would therefore be prudent to expose male and female rats to ISO or ENR in future studies to deduce in more detail how positive or harmful life events influence social interaction and potential anhedonia phenotypes in adulthood, and whether the aberrant social behavior observed in Study I and II can be rescued by the beneficial effects of environmental ENR.

Conversely, stronger effects of *Cacna1c* genotype on learning and memory may be obtained by changing parameters of the cognitive paradigms themselves to reflect varying difficulty levels, like more remote probe trials in the RAM (White et al., 2008) or a longer ITI during NOR (Temme et al., 2016). Such behavioral studies will provide important clarification of the relationship between *Cacna1c* influence and cognitive demand. Likewise, even though our approach holds the advantage of proving long-term effects of ISO and ENR, future experimenters may want to consider testing the cognitive abilities in animals fresh out of environmental housing to control for potential rescue effects. The postulated protective influence of *Cacna1c* in older rodents (Zanos et al., 2015) also seems to be a promising avenue to an understanding of how *Cacna1c* affects individuals across the lifespan in a sex-dependent manner. Additionally, since physiological indicators have been suggested to be more sensitive than behavioral measures (Gurung & Prata, 2015), future studies are encouraged to extend the scope of data acquisition to e.g. the examination of the amygdala, as well as BDNF levels and dopamine signaling in the VTA and PFC, to shed more light on the effect of *Cacna1c* on underlying neurobiological mechanisms and help bridge the gap between genes, early experiences and behavior across species (Short & Baram, 2019).

In summary, a partial ablation of the *Cacna1c* gene and the housing manipulations employed in Study I-IV clearly alter disorder-relevant outcomes, proving that rat models which capitalize on the diverse behavioral assays of the species, new technologies for genetic manipulation and the opportunity to uncover GxE interactions in controlled laboratory settings can elucidate the main effects of gene and environment, as well as their interplay on intermediate phenotypes. At present, the translational value *Cacna1c* rodent studies can provide is encumbered by the fact that most studies focus on male animals and thus restrict sex comparisons and generalizability to disorders, especially those with strong female prevalence.

Research on clearly defined intermediate phenotypes, such as in Study I-IV, can be a useful tool to aid in the elucidation of disease pathology by creating more homogenous subgroups across disorders, and thus help to reduce limitations imposed by current diagnostic boundaries. Of course, translational animal models are only ever an approximation of specific facets of human ailments and one model is unlikely to capture a disease or associated phenotype in all aspects. Regarding the development of new treatments for human mental disorders, the "best experimental model is man" (Millan et al., 2012, p. 151) – in theory. Unfortunately, the question of whether risk variants in *CACNA1C* result in over- or underexpression of *CACNA1C* mRNA in the brain can only be answered postmortem (Bigos et al., 2010; Gershon et al., 2014) and whether this, in turn, leads to a gain or loss of function in calcium channels needs to be clarified before effective and reliable LTCC-modifying drugs can be developed (Berger & Bartsch, 2014; Casamassima et al., 2010). In rodents, the TS2-neo model clearly provides evidence of a gain of function in social and cognitive deficits, but concomitant findings in *Cacna1c* knockout models showcase the complexity of this mechanism. In the end, even though not all signs point towards a decreased $Ca_v1.2$ functionality, the results from Study I-IV indicate that a loss of function is definitely somehow involved in impaired social behavior and, to a lesser degree, in aberrations of cognitive function as seen in our constitutive *Cacna1c* haploinsufficient rats. As even longitudinal deep-phenotyping studies of relevant animal models have their limitations, though, the research conducted in our lab is only one part of a bigger interdisciplinary approach to identify the role of *CACNA1C* in neuropsychiatric disorders with clinical, translational and molecular methods informing one another to validate potential genetic associations with intermediate phenotypes in brain structure, function and behavior (Kircher et al., 2018).

All things considered, the investigation of genetic and environmental influence on behavioral domains is not only essential to get a better grasp on how social and cognitive deficits arise in selected vulnerable individuals harboring genetic risk, or after certain adverse life events, but also to create better evidence-based treatment methods which may help rectify or at least ameliorate these impairments so patients are better equipped to handle the challenges of daily life, which, in turn, can increase the likelihood of symptom remission (Millan et al., 2012). By deciphering the etiology of disorder-associated intermediate phenotypes via translational research, we can help pave the way to a more holistic diagnostic system, enhanced treatment options for diagnosed patients (Gottesman & Gould, 2003), as well as to the identification of at-risk individuals that may benefit from early intervention.

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Erklärung

Hiermit versichere ich, dass ich die vorliegende Dissertation

"Effects of *Cacna1c* Haploinsufficiency and Environmental Impact on Spatial Learning, Cognitive Flexibility and Social Behavior 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.

_____ _____

Ort, Datum Moria Braun