ANGST UND FURCHT IN DER RATTE

— über den Einfluss situationsspezifischer und biologischer Faktoren auf das Spektrum der Ultraschallvokalisationen —

Dissertation

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INHALT

VORGELEGTE ARBEITEN – ÜBERSICHT

STUDIE I:

Willadsen M, Best LM, Wöhr M, & Clarke PBS (2018). Effects of anxiogenic drugs on the emission of 22-and 50-kHz ultrasonic vocalizations in adult rats. *Psychopharmacology*, 235(8), 2435-2445.

STUDIE II:

Fendt M, Brosch M, Wernecke KE, **Willadsen M**, & Wöhr M (2018). Predator odour but not TMT induces 22-kHz ultrasonic vocalizations in rats that lead to defensive behaviours in conspecifics upon replay. *Scientific reports*, 8(1), 1-11.

STUDIE III:

Willadsen M, Uengoer, M, Schwarting RKW, Homberg JR, & Wöhr M (2021). Reduced emission of alarm 22-kHz ultrasonic vocalizations during fear conditioning in rats lacking the serotonin transporter. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, 108, 110072.

STUDIE IV:

Willadsen M, Uengoer M, Sługocka A, Schwarting RKW, Homberg JR, Wöhr M (2021). Fear Extinction and Predictive Trait-Like Inter-Individual Differences in Rats Lacking the Serotonin Transporter. *International Journal of Molecular Sciences* 22 (13), S. 7088.

ZUSAMMENFASSUNG

Für das Verständnis pathologischer Angstzustände ist eine änderungssensitive Modellierung von Angst und Furcht in entsprechenden Modellorganismen unerlässlich. In Ratten wird die Emission von Ultraschallvokalisationen (USV) gemeinhin als Ausdruck emotionaler Erregung verstanden, der an unterschiedlich valente Situationen gekoppelt ist. Hierbei werden appetitive Situationen wie Spielverhalten, Paarung und die Gabe von Suchtmitteln mit Vokalisationen in einem Frequenzbereich von um die 50 kHz assoziiert. Aversive Zustände, wie die akute Bedrohung durch Fressfeinde oder ähnlich bedrohliche Manipulationen im Labor werden hingegen mit Vokalisationen in einem Frequenzbereich von um die 22 kHz in Verbindung gebracht. Es wird vermutet, dass sich die Produktion von 50- und 22-kHz USV gegenseitig ausschließt, da sie von grundverschiedenen, neuronalen Mechanismen gesteuert werden, was die Betrachtung von 50-kHz USV in aversiven Kontexten bis jetzt weitgehend ausschloss. Eine klare Dichotomie der USV-Produktion lässt sich jedoch nicht aufrechterhalten, da sowohl inhärent appetitive Situationen durchaus von 22-kHz USV begleitet werden können, während 50-kHz USV ebenso in nicht explizit belohnenden oder sozialen Kontexten emittiert werden. Stattdessen soll gezeigt werden, dass sich USV-Emission in einem Spektrum vollzieht, das die Produktionen von 50- oder von 22-kHz USV in Abhängigkeit von situationsspezifischen und biologischen Faktoren wahrscheinlicher macht. Von besonderem Interesse ist in diesem Zusammenhang die Betrachtung von Angst und Furcht als distinkte Phänomene, die in unterschiedlicher Weise Einfluss auf das Spektrum der USV nehmen. Angst wird hierbei als Reaktion auf ungewisse und mehrdeutige Situationen verstanden, während Furcht als Antwort auf realistische, oft imminente Bedrohung durch spezifische Stimuli mit entsprechenden diskreten Verhaltensantworten definiert wird.

In den vorliegenden Studien wurden verschiedene Methoden, Apparaturen und Tests verwendet, um die situationsspezifischen Einflüsse auf die USV-Emission untersuchen zu können. Standardprozeduren zur Messung von angstähnlichem Verhalten (erhöhtes Plus-Labyrinth) und konditionierter Furcht wurden durch die Konfrontation mit Raubtiergeruch und die pharmakologische Manipulation des affektiven Zustands in neutraler Umgebung ergänzt. Zudem wurde der Einfluss biologischer Faktoren – wie Geschlecht – und modifizierter neuronaler Transmission mittels genetischer Manipulation des serotonergen Systems, untersucht. Vor dem Hintergrund von Angst und Furcht wurden ebenso zeitlich stabile Merkmale (*Traits*) in Abhängigkeit der biologischen Faktoren betrachtet.

Es zeigte sich, dass Angst prinzipiell mit einer Verringerung der 50-kHz USV einhergeht, während die Produktion von 22-kHz USV ausschließlich in Verbindung mit konkreten,

furchtinduzierenden Stimuli auftraten. Mit Hilfe des USV-Spektrums lässt sich eine Verschiebung der USV-Emission in Abhängigkeit von Geschlecht und serotonerger Transmission zeigen. Hierbei verschieben sich die Prävalenzen für 50- und 22-kHz USV über verschiedene Situationen hinweg, da sowohl Weibchen als auch genetisch manipulierte Ratten in neuen, ungewohnten Umgebungen mehr 50-kHz USV produzieren, deren Emission mit zunehmender Aversivität der Situation länger beibehalten, und in Konfrontation mit akuter Bedrohung gleichsam weniger 22-kHz USV produzieren. Die geschlechtsabhängige Verschiebung der USV wird von einer verminderten Ängstlichkeit über verschiedene Tests hinweg begleitet. Der Einfluss serotonerger Transmission auf die Rufproduktion ist allerdings gegenläufig zu den lokomotionsbasierten Maßen von Angst und Furcht.

Die Evaluation der USV-Emissionen anhand des vorgestellten Spektrums kann demnach zur gezielten Differenzierung von Angst und Furcht dienen und erlaubt somit, lokomotionsbasierte Verhaltensweisen mit Hilfe feinerer Nuancen besser interpretieren zu können. Situationsspezifische Gegebenheiten führen hierbei zu einer angstinduzierten Abnahme der 50-kHz USV oder einer furchtinduzierten Zunahme von 22-kHz USV. Biologische Faktoren wie Geschlecht, veränderte serotonerge Transmission oder allgemeine Ängstlichkeit moderieren zudem den situationsspezifischen Einfluss auf das Spektrum der USV.

1 Einleitung

Angst und Furcht sind hoch adaptive Phänomene, die komplexe Reaktionen des Organismus hervorrufen und in ihrem Ausdruck hoch variabel sind (Blanchard et al. 2008). Von besonderem Interesse sind hierbei die pharmakologischen Grundlagen generellen und pathologischen Angst- und Furchtverhaltens (Blanchard et al. 2008), für deren Erforschung Tiermodelle unerlässlich sind (Homberg et al. 2021). Ratten – als Modellorganismen – zeigen ein vielfältiges Verhaltensrepertoire, zu dem neben diversen beobachtbaren Verhaltensweisen, die Emission von pfeifenartigen Rufen im Ultraschallbereich, sogenannten Ultraschallvokalisationen (USV), zählt. Sie werden weithin als Ausdruck emotionaler Erregung verstanden und eignen sich als Instrument zur Modellierung typischer und pathologischer Emotionen bei Nagetieren (Brudzynski 2021). USV sind im Zusammenhang mit der Untersuchung von Angst und Furcht von besonderem Interesse und sollen im Folgenden näher beschrieben werden. Abhängig von Alter, situativen Faktoren und affektivem Zustand werden USV in drei Kategorien unterteilt.

1.1 Ultraschallvokalisationen (USV)

Jungtiere emittieren in potenziell lebensbedrohlichen Situationen, wie beispielsweise der Trennung vom Muttertier oder dem Wurf, isolationsinduzierte Rufe im Bereich von 40-kHz (Hofer 1996; Allin und Banks 1971; Hofer und Shair 1978). Diese Rufe wiederum lösen im Muttertier Such- und Eintrageverhalten aus (Allin und Banks 1972).

Im Gegensatz zu Jungtieren unterscheidet man bei juvenilen und adulten Tieren zwei generelle Ruftypen, abhängig von Frequenz und Länge: kurze, hochfrequente 50-kHz USV und lange, niederfrequente 22-kHz USV. 50-kHz USV sind gemeinhin mit appetitiven Situationen (Brudzynski 2007, 2013, 2015, 2021), wie Spiel, Paarung oder Selbststimulation des Gehirns assoziiert; wohingegen 22-kHz USV vor allem in aversiven Situationen auftreten, wie beispielsweise bei Verabreichung eines elektrischen Schocks (Borta et al. 2006; Kaltwasser 1991; Wöhr et al. 2005), bei Entzugserscheinungen (Covington und Miczek 2003) oder während aggressiven Interaktionen mit Artgenossen (Sales 1972) und der Konfrontation mit Fressfeinden (Blanchard et al. 1991b; Blanchard und Blanchard 2008).

1.1.1 50-kHz USV

50-kHz USV werden mit positiven affektiven Zuständen assoziiert und erfüllen eine pro-soziale, kommunikative Funktionen. Der Paarungserfolg adulter Tiere wird maßgeblich von der Emission von 50-kHz USV bestimmt. Ohne die Fähigkeit des männlichen Tieres, 50-kHz USV auszustoßen, verringert sich die Bereitschaft des Weibchens zur Kopulation (Thomas et al. 1981; Thomas et al. 1982; White und Barfield 1990; White et al. 1990). Werden jedoch vor oder während des Paarungsversuchs von zuvor devokalisierten Männchen 50-kHz USV abgespielt, erreicht die sexuelle Bereitschaft des Weibchens das typische Niveau (White und Barfield 1990; Geyer et al. 1978). Eine Präferenz der weiblichen Tiere für 50-kHz USV zeigt sich ebenso in nicht-sexuellen Kontexten, in denen weibliche Ratten 50-kHz USV gegenüber Stille (Barfield et al. 1979) oder anderen akustischen Signalen (Willadsen et al. 2014) bevorzugen.

Neben der Sicherung des Paarungserfolges dienen 50-kHz USV auch anderen pro-sozial kommunikativen Funktionen in nicht-sexuellen Interaktionen wie beispielsweise dem juvenilen Spiel [*rough-and-tumble Play*] (Burgdorf et al. 2008; Knutson et al. 1998; Himmler et al. 2014; Kisko et al. 2015). Sowohl während der interspezies-spezifischen Interaktion als auch in Antizipation dessen (Himmler et al. 2014; Kisko et al. 2015) können vermehrt 50-kHz USV beobachtet werden. Artgenossen, die eine hohe Anzahl an 50-kHz USV ausstoßen, werden als Spielpartner bevorzugt (Panksepp et al. 2002), während das Spielverhalten devokalisierter Ratten im Vergleich beeinträchtigt ist (Kisko et al. 2015).

Wird das Spielverhalten über die spezies-spezifische Interaktion hinaus durch einen humanen Experimentator nachgeahmt, sind ähnliche Effekte auf das Rufverhalten der Tiere zu verzeichnen. Werden juvenile Ratten in standardisierter Weise durch eine menschliche Hand „gekitzelt“ [T*ickling*], werden ebenfalls vermehrt 50-kHz USV ausgestoßen (Burgdorf und Panksepp 2001; Mällo et al. 2007a; Schwarting et al. 2007; Wöhr et al. 2009). Tiere, die diese Prozedur als besonders belohnend empfinden und sich entsprechend schnell der kitzelnden Hand zuwenden, stoßen mehr 50-kHz USV aus als deren weniger interessierte Artgenossen (Panksepp und Burgdorf 2000; Panksepp und Burgdorf 2003). Somit können 50-kHz USV neben ihrer pro-sozialen Kommunikationsfunktion als Indikator eines positiven affektiven Zustands dienen.

Die Präferenz für pro-soziale Kommunikationssignale lässt sich über das Paarungs- und Spielverhalten hinaus beobachten. Werden Tiere in einem vermeintlich nicht-sozialen Kontext mit einer 50-kHz USV-Aufnahme konfrontiert, zeigen sie auch hier Annäherungsverhalten an die Geräuschquelle (Willuhn et al. 2014; Wöhr und Schwarting 2007, 2009, 2012; Schwarting et al.

2018). Hierbei scheinen vor allem den 50-kHz USV inhärente Charakteristika ausschlaggebend, da den Rufen ähnliche Töne kein explizites Annäherungsverhalten auslösen.

Die positiv affektive Funktion zeigt sich ebenfalls auf hirnorganischer Ebene. So führt beispielsweise das Abspielen von 50-kHz USV zu einer vermehrten Dopaminausschüttung im Bereich des Nucleus Accumbens (Willuhn et al. 2014) – einem mit Belohnung assoziierten Areal (Volkow und Morales 2015; Volkow et al. 2019; Gardner 2011). Wird dieses Areal wiederum stimuliert, kommt es auch hier zu einer vermehrten Produktion von 50-kHz USV (Burgdorf et al. 2001). Neben der gezielten Applikation in spezifische Hirnareale, führt die systemische Applikation dopaminerger Wirkstoffe, wie Amphetamin, zu einer erheblichen Steigerung der Rufproduktion (Ahrens et al. 2009; Burgdorf et al. 2001; Natusch und Schwarting 2010; Pereira et al. 2014; Rippberger et al. 2015) – und einer merklichen Veränderung des Rufprofils (Wright et al. 2010).

Die Klasse der 50-kHz USV zeichnet sich durch kurze (30-50ms) pfeifenähnliche Rufe im Frequenzbereich zwischen 32 und 96 kHz aus, die im Allgemeinen sehr heterogen ist und sich in Abhängigkeit vom Subtyp in neurochemischer Hinsicht und behavioraler Bedeutung unterscheidet. Je nach Beschaffenheit und Detailreichtum der Differenzierung können bis zu vierzehn Subtypen unterschieden werden (Wright et al. 2010). Die gröbste Unterteilung findet anhand des Vorhandenseins von Frequenzunterschieden (Modulation) innerhalb eines Rufes statt. Wobei unterschieden wird zwischen Rufen, die in ihrer Frequenz variieren (frequenzmoduliert, FM) oder keinerlei Modulation aufweisen (flach). Die Zusammensetzung des Rufprofils hängt von verschiedenen Faktoren ab. Flache Rufe sind vermehrt in sozialen Kontexten zu finden sind und werden während einer kurzen Phase sozialer Isolation von den Tieren produziert (Wöhr et al. 2008). Da sowohl das dem Gruppenkäfig entnommene Testtiere als auch deren verbleibenden Artgenossen flache Rufe produzieren, wird angenommen, dass sie der Wiederherstellung und Erhaltung von Sozialkontakt dienen (Wöhr et al. 2008). Frequenzmodulierte 50-kHz USV hingegen sind mit Belohnung assoziiert und werden beispielsweise in Versuchen zur Selbstadministration bevorzugt emittiert (Burgdorf et al. 2007). Ihr Auftreten ist stark an die dopaminerge Transmission gekoppelt (Wintink und Brudzynski 2001). Die selektive Zerstörung von Projektionsbahnen führt zu einem Absinken der FM 50-kHz USV (Ciucci et al. 2009); während Dopaminagonist Amphetamin das Rufprofil zugunsten der FM 50-kHz USV verschiebt (Wright et al. 2010; Simola 2015).

1.1.2 22-kHz USV

Im Gegensatz zu 50-kHz USV sind 22-kHz USV größtenteils mit aversiven Situationen assoziiert und es wird angenommen, dass sie einen „negativen affektiven Zustand ähnlich Angst und Furcht“ (Wöhr und Schwarting 2013) widerspiegeln. Diese Rufe sind sowohl in naturalistischen Situationen als auch unter verschiedenen Laborbedingungen beobachtbar. Dazu gehören aggressive Interaktionen mit Artgenossen (Sales 1972; Vivian und Miczek 1993) und der Kontakt mit Raubtieren (Blanchard und Blanchard 2008; Blanchard et al. 1991a; Blanchard et al. 1991b). Es wird angenommen, dass 22-kHz USV als Alarmrufe fungieren um Artgenossen vor Bedrohungen zu warnen. In einer Reihe von Experimenten zum Abwehrverhalten gegenüber Raubtieren in sozialen Gruppen von Ratten, die in einem sichtbaren Höhlensystem leben, zogen sich Ratten in Konfrontation mit einer Katze in ihren Bau zurück und begannen dort, 22-kHz USV auszusenden (Blanchard et al. 1990; Blanchard et al. 1991b). Es gibt Hinweise, dass diese 22-kHz USV-Emissionen durch die Anwesenheit von Artgenossen verstärkt werden kann und eine Furchtreaktion in selbigen ausgelöst. Letzteres wurde sowohl bei der sozialen Furchtkonditionierung beobachtet (Kim et al. 2010; Wöhr und Schwarting 2008b) als auch bei der Präsentation von 22-kHz USV unter Laborbedingungen, die eine Verhaltenshemmung bei Empfängern auslöst (Brudzynski und Chiu 1995; Wöhr et al. 2020).

Im Labor werden 22-kHz USV typischerweise durch die Verabreichung von Luftstößen hervorgerufen (Brudzynski und Holland 2005; Inagaki und Mori 2014), durch akustische Schreckreize (Kaltwasser 1991; Vivian et al. 1994) oder durch elektrische Stimulation (Cuomo et al. 1988; Tonoue et al. 1986) evoziert und dient oft als zusätzliches Maß in Experimenten zur Furchtkonditionierung (Molewijk et al. 1995b; Rowan et al. 1990). Die Stärke der 22-kHz USV-Emissionen hängt hierbei mit der Aversivität der Situation zusammen. Höhere Intensitäten elektrischer Stimulation gehen dementsprechend mit einer vermehrten 22-kHz USV-Produktion und insgesamt lauteren Rufen einher (Wöhr et al. 2005). Eine potenzierte 22-kHz USV-Emission wird bei Ratten beobachtet, die sich entweder durch höhere Angstmerkmale auszeichnen (Borta et al. 2006), oder nach pränataler Immunaktivierung (Yee et al. 2012a), mütterlicher Vernachlässigung (Wöhr und Schwarting 2008a) und Exposition von Stress in der Adoleszenz (Yee et al. 2012b).

Die Emission von 22-kHz USV wird gemeinhin mit der mesolimbisch cholinergen Signaltransduktion assoziiert (Brudzynski 2019, 2021; Brudzynski et al. 2018), speziell mit nahezu allen Bestandteilen der Amygdala und des Periaquäduktalen Grau (PAG) (Choi und Brown 2003; Yajima et al. 1980). Die Stimulation cholinerger Projektionen führt zu einer weitreichenden

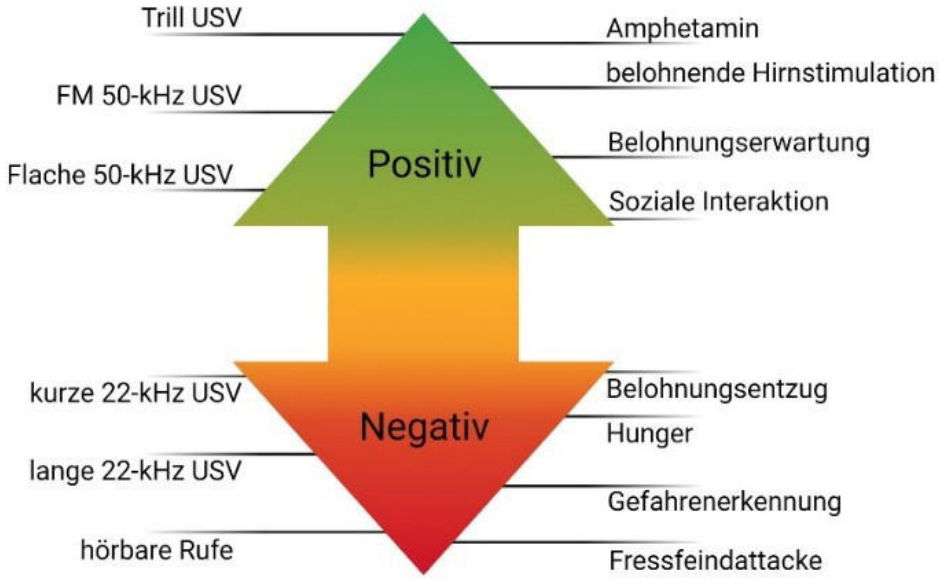
Verhaltensantwort, die auf einen negativen affektiven Zustand hindeutet und mit einer erhöhten 22-kHz USV-Produktion einhergeht (Bihari et al. 2003; Brudzynski et al. 1991; Brudzynski 1994; Brudzynski und Barnabi 1996; Brudzynski et al. 2018), während die Entfernung der basolateralen (BLA) oder zentralen Amygdala (CeA) die Emission der 22-kHz USV unter aversiven Bedingungen unterdrückt (Choi und Brown 2003; Koo et al. 2004).

22-kHz USV sind verhältnismäßig lang (300-4000ms) und treten vornehmlich in einem Frequenzbereich zwischen 18 und 32 kHz auf. Da die Gruppe der 22-kHz USV im Vergleich zu 50-kHz USV extrem homogen ist, werden sie auf ihre temporalen Muster hin untersucht, indem sie zu sogenannten *Bouts* zusammengefasst werden. Basierend auf detaillierten Studien über die Struktur von 22-kHz USV-Emissionen (Miczek und van der Poel 1991; van der Poel et al. 1989) wurde ein *Bout* als ein Ruf oder eine Anzahl von Rufen definiert, die von anderen Rufen durch Inter-Bout-Intervalle von mehr als 320 ms getrennt sind. Um die zeitliche Strukturierung der 22-kHz USV-Produktion zu beschreiben, wurden die Anzahl der Durchgänge und die Anzahl der Rufe innerhalb eines Durchgangs, d. h. die Durchgangslänge, bestimmt, da zuvor gezeigt wurde, dass die zeitliche Strukturierung von der Aversivität der Situation abhängt (Miczek und van der Poel 1991), durch frühen Lebensstress beeinflusst wird (Yee et al. 2012a) und durch Anxiolytika moduliert wird (van der Poel et al. 1989). Zusätzlich zu den typischen „langen“ 22-kHz USV wurden zudem kurze 22-kHz USV berichtet (Brudzynski et al. 1993). Niedrige, kurze 22-kHz USV wurden als Rufe mit einer Spitzenfrequenz unter 32 kHz und einer Rufdauer von weniger als 300 ms definiert, wobei ihre verhaltensbezogene Bedeutung noch nicht bestimmt wurde (Brudzynski 2013, 2019). Es wird jedoch vermutet, dass sowohl kurze als auch lange 22-kHz USV mit Aversion assoziiert sind (Barker et al. 2010) und im Zusammenhang damit diskutiert, ob kurze 22-kHz USV eher eine „innere Unzufriedenheit“ (d. h. ohne äußere Gefahr oder Bedrohung) darstellen, während sich lange 22-kHz USV gegen externale, reale Bedrohung richten (Brudzynski 2015).

1.1.3 Systematik der USV auslösenden Situationen

Verschiedene Autor:innen schlagen vor, die beiden Rufarten der 50- und 22-kHz USV aufgrund ihrer neuronalen Korrelate, strikt zu trennen (Barker 2018; Brudzynski 2019, 2021). Die Dichotomie aversiven und appetitiven Verhaltens gründet sich in den damit assoziierten, distinkten Neurotransmittersystemen, die ebenfalls die USV-Emission steuern und somit

Abbildung 1. Dichotomie USV nach Barker (2018).



Anmerkung. In Abhängigkeit des emotionalen Zustands (positiv oder negativ) vokalisieren Tiere entweder 50-kHz USV oder 22-kHz USV. Abgebildet sind die jeweiligen Rufarten mit assoziierten Situationen, in denen sie auftreten.

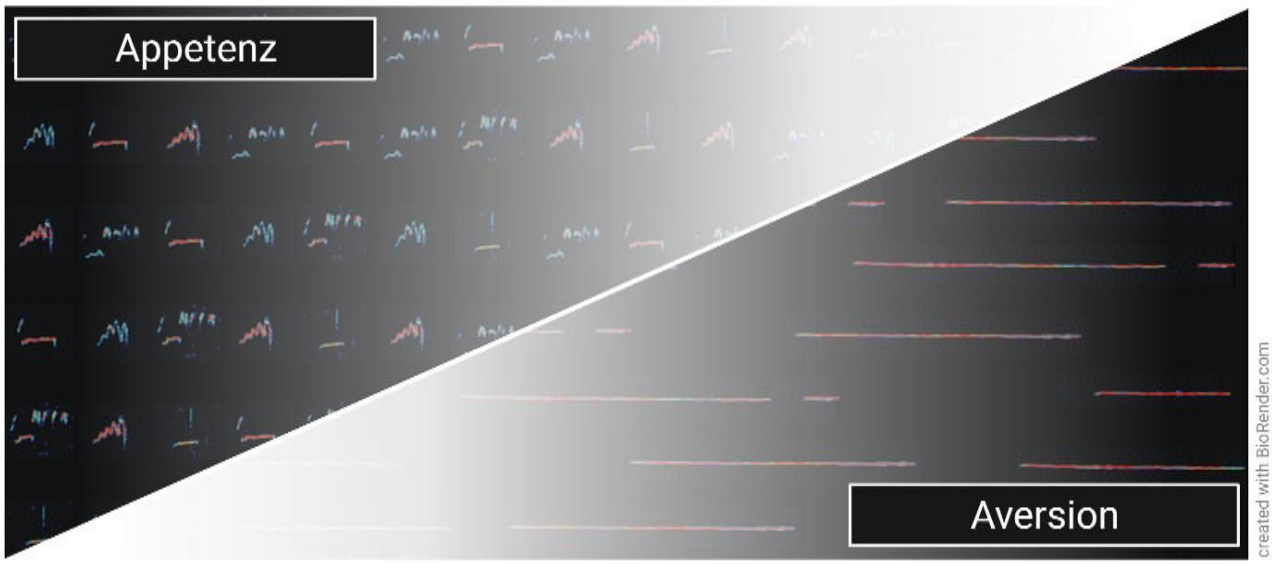
einander ausschließende Systeme der Verhaltensregulation darstellen. Appetitive Situationen, wie Suchtmittel [*Drugs of Abuse*], Belohnung und soziale Interaktion, seien demnach mit dem mesolimbisch-dopaminergen System und dadurch mit 50-kHz USV assoziiert; aversive Bedingungen, wie Belohnungsentzug oder Gefahr, seien hingegen vornehmlich mit mesolimbisch-cholinergen Prozessen und dementsprechend mit 22-kHz USV assoziiert (siehe Abbildung 1).

Neben der dichotomen Einteilung von 50-kHz USV in appetitiven und 22-kHz in aversiven Situationen, gibt es jedoch Hinweise, dass ebenso in neutralen, weder mit Belohnung noch Sozialkontakt assoziierten, Situationen 50-kHz USV gemessen werden. In Untersuchungen zu pharmakologisch potenzierenden Effekte auf 50-kHz USV werden gleichwohl in den Kontrollbedingungen – ohne pharmakologisch wirksame Substanzen – 50-kHz USV detektiert (Engelhardt et al. 2017; Knutson et al. 1999; Pereira et al. 2014; Rippberger et al. 2015; Thompson et al. 2006; Wintink und Brudzynski 2001; Wöhr et al. 2014). Wobei das Vorhandensein von Einstreu in einer neutralen Testumgebung einen verstärkenden Effekt auf die 50-kHz USV-Emission hat (Brenes et al. 2016; Natusch und Schwarting 2010).

Ebenso können komplexe Verhaltensabläufe und soziale Interaktionen von 50- und 22-kHz USV gleichermaßen begleitet werden. Obwohl der Paarungserfolg, wie beschrieben, prinzipiell mit dem Vorhandensein von 50-kHz USV assoziiert ist, emittieren Männchen in der postejakulatorischen

Refraktärzeit ebenso 22-kHz USV (Barfield et al. 1979; Burgdorf et al. 2008). Auch in aggressiven Interaktionen wurden ambivalente USV-Emissionen berichtet. In Konfrontation mit einem bedrohlichen Artgenossen stoßen männliche Ratten sowohl 50- als auch 22-kHz nach erfolgter Attacke aus (Vivian und Miczek 1993). Bei näherer Betrachtung verschiedener Komponenten aggressiven Verhaltens, wird gegenseitiges Beschnüffeln und Attackieren von 50-kHz USV begleitet, wohingegen Annäherungsverhalten mit 50- aber auch 22-kHz USV-Emission einhergeht (Sales 1972). Auch vermeintlich appetitive Situationen können sowohl 50- als auch 22-kHz USV-Emissionen evozieren. Verschiedene Untersuchungen zur Interspezies-Interaktion messen beispielsweise beide Rufarten während der *Tickling*-Prozedur (Mällo et al. 2007a; Popik et al. 2012; Popik et al. 2014; Wöhr et al. 2009). Ob es sich hierbei um intraindividuelle Schwankungen handelt und ein einzelnes Tier somit zwischen appetitivem und aversivem Zustand wechselt, oder ob hier interindividuelle Unterschiede in der Wahrnehmung des *Tickling* vorliegen, ist aus den vorliegenden Befunden nicht ersichtlich. Dennoch werfen beide denkbaren Szenarien die Frage auf, unter welchen Bedingungen eine vermeintlich standardisierte Laborsituation die Emission von 50- oder 22-kHz USV begünstigt. Ob es einen Scheitelpunkt zwischen den Rufarten gibt, an dem der Übergang von 50-kHz USV zu 22-kHz USV liegen könnte, ist derzeit wenig untersucht. Darüber hinaus gibt es bereits Hinweise auf einen sogenannten "ambivalenten Zustand", der mit einer Rufproduktion ähnlich der kurzen 22-kHz USV einhergeht, aber in der Situation sozialen Annäherungsverhaltens zu beobachten ist (Berz et al. 2021a; Berz et al. 2021b). Hierbei reagieren Ratten auf die Wiedergabe natürlicher 50-kHz USV eines Artgenossen mit sozialem Annäherungsverhalten und sogenannten „Antwortrufen" [*Response Calls*]. Diese Rufe sind

Abbildung 2. Spektrum der USV.



prinzipiell keiner der beiden Rufarten zuzuordnen, da sie in Länge und Struktur den 50-kHz USV ähneln, sich jedoch im Frequenzbereich der 22-kHz USV bewegen. Die USV-Emission gleicht somit weniger einer klaren Dichotomie, sondern ist als ein Spektrum zu verstehen, auf dem – je nach situativen Bedingungen und biologischen Faktoren – die Auftretenswahrscheinlichkeit für die unterschiedlichen Rufarten variiert (siehe Abbildung 2). Wichtig ist hierbei, dass inhärent appetitive Situationen eine Produktion von 50-kHz USV wahrscheinlicher machen, während (einige) aversive Situationen mit einer höheren Wahrscheinlichkeit für die Emission von 22-kHz USV einhergehen.

1.2 Situationsspezifische Faktoren – Angst und Furcht

Übermäßige Angst und Furcht sind Kennzeichen einer Reihe psychischer Störungen, insbesondere von Angststörungen, einschließlich Phobien und posttraumatischer Belastungsstörung (PTBS; Diagnostic and statistical manual of mental disorders (5th Ed) 2013). Es wird vermutet, dass solche neuropsychiatrischen Störungen mit einer unzureichenden Löschung von Furchterinnerungen (Extinktion) verbunden sind (VanElzakker et al. 2014). Extinktion ist die Hemmung konditionierter Furchtreaktionen, die normalerweise als Folge der wiederholten Exposition gegenüber einem konditionierten Reiz (*conditioned* stimulus; CS) in Abwesenheit eines aversiven unkonditionierten Reizes (*unconditioned* stimulus; US) auftritt (Vervliet et al. 2013; Lonsdorf et al. 2017) – näheres siehe Kapitel 1.2.3 Furchtkonditionierung. Erfolgreiche Extinktion ist daher eine Schlüsselkomponente der weit verbreiteten Expositionstherapie bei der Behandlung von Angststörungen (Furini et al. 2014). Stress und Umweltfaktoren können jedoch die Extinktion und somit die Wirksamkeit der Expositionstherapie hemmen (Maren und Holmes 2016). Um die dahinterliegenden neurobiologischen Mechanismen von Angst und Furcht zu ermitteln, sind Tiermodelle unerlässlich (Homberg et al. 2021). Hierbei ist zwischen zwei groben Kategorien der Tiermodelle zu unterscheiden (Steimer 2002): Auf der einen Seite stehen Tests, die einen akuten angst- oder furchtähnlichen Zustand evozieren sollen [S*tate Anxiety*] und somit sowohl unkonditionierte Reaktionen als auch erlernte Furcht modellieren. Auf der anderen Seite werden charakterähnliche Angstneigung oder pathologische Angst [*Trait Anxiety*, siehe Kapitel 1.3.3] beispielsweise mit Hilfe von Modellen untersucht, die durch selektive Züchtung oder anderweitige genetische Manipulation abweichendes Angst- und Furchtverhalten aufweisen. Als ökologisch valide und von hohem translationalen Wert gelten hierbei das erhöhte Plus-Labyrinth (Lister 1990; siehe Kapitel 1.2.2) und die Pawlow'sche Furchtkonditionierung (Lonsdorf et al. 2017; siehe Kapitel 1.2.3). Auch

andere angstauslösende Situationen, wie die unkonditionierte Reaktion auf Raubtiergeruch (Kapitel 1.2.4) oder pharmakologische Manipulationen (Kapitel 1.2.5) können neben diesen weitverbreiteten Tests in Bezug auf deren Anxiogenese untersucht werden. Angst und Furcht werden hierfür als distinkte Phänomene betrachtet und sollen am Beispiel des *Predatory Imminence Continuum* (Fanselow und Lester 1988; Kapitel 1.2.1) veranschaulicht werden.

1.2.1 Predatory Imminence Continuum

Die Hauptfunktion von Angst und Furcht bestehe gleichermaßen darin, „als Signal für Gefahr [oder] Bedrohung zu agieren und angemessene Anpassungsreaktionen auszulösen“ (Steimer 2002) und wird in diesem Sinne teilweise mit identischen Verhaltensmaßen, wie Inaktivität erfasst. Die meisten Konzepte, die Angst und Furcht voneinander unterscheiden, definieren Angst als ungewiss und mehrdeutig, während Furcht als Antwort auf reale, oft imminente Bedrohung mit entsprechenden diskreten Verhaltensantworten verstanden wird (Blanchard und Blanchard 2008). Angst wird hier als internaler Zustand anhaltender Wachsamkeit [Q*uality of sustained Vigilance*; LeDoux 2015] beschrieben, also als generalisierte Antwort auf unbestimmte Gefahren (Steimer 2002). Furcht hingegen fokussiere sich auf externe Bedrohungen (Steimer 2002), auf spezifische, präsente Hinweisreize, die eine gewisse Dringlichkeit [*Quality of Emergency*; (LeDoux 2015)] evozieren. Diese Dringlichkeit der Furcht bildet also einen zentralen motivationalen Zustand, der direkt eine Abwehrreaktion provoziert LeDoux 2015.

Die Theorie Fanselows (1988) über das „Kontinuum der drohenden Gefahr (eines Fressfeindes)“ [P*redatory Imminence Continuum*] verfolgt einen funktional behavioristischen Ansatz und ist in der Lage, Angst und Furcht konzeptuell voneinander zu trennen. Die Umwelt eines Tieres, das potenziell Beute eines Raubtieres ist, wird hierbei als Umgebung zu lösender Probleme verstanden; Probleme, die den Fortpflanzungserfolg des potenziellen Beutetieres unterminieren können. Hierzu zählt vor allem der mögliche Kontakt zu Fressfeinden, den es im Sinne des Fortpflanzungserfolges unbedingt zu vermeiden gilt. Abhängig von der räumlichen und zeitlichen Nähe zum Fressfeind wird unterschiedliches Abwehrverhalten gezeigt, welches die aktuellen Situationen für das Tier bestmöglich auflösen sollen. Das dadurch entstehende Kontinuum richtet sich sowohl nach der physiologischen als auch der psychologischen Nähe zur Bedrohung (siehe Abbildung 3):

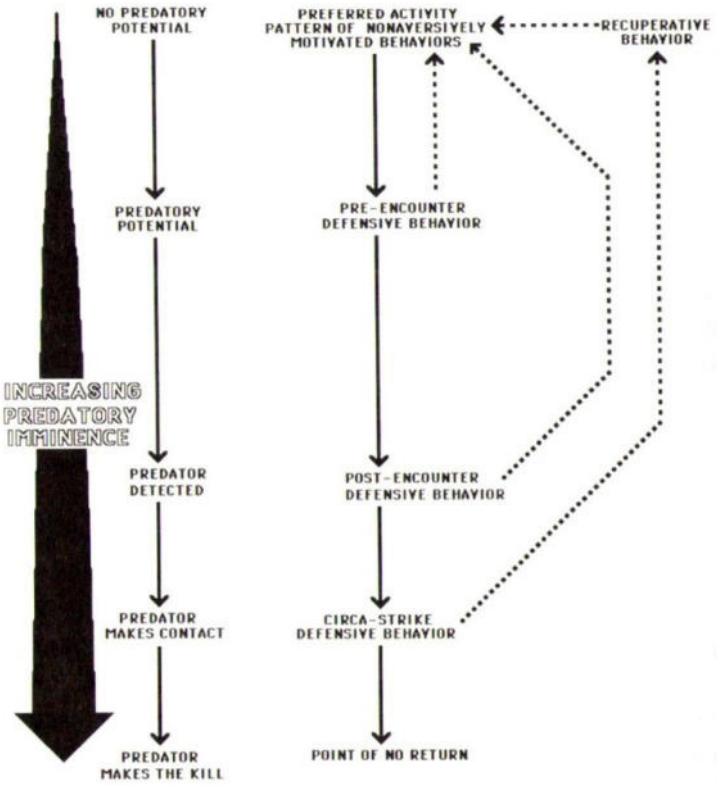
1 Geht von der Situation keinerlei Gefahr eines Kontakts aus, wie beispielsweise im Bau eines Nagetieres, wird kein Abwehrverhalten gezeigt und stattdessen bevorzugten Verhaltenmustern [*Preferred Activity Pattern*], wie Nahrungsaufnahme, nachgegangen.

2 Ist eine Begegnung im Bereich des Möglichen, etwa bei der Futtersuche in offenem, unbekanntem Terrain, passt das Beutetier die eigenen Aktivitäten an, um die Wahrscheinlichkeit einer Begegnung zu minimieren. Diese prä-Begegnungsphase [P*re-encounter Phase*] ist durch vermehrtes Explorations- und Risikobewertungsverhalten gekennzeichnet.

3 Wird eine tatsächliche Gefahr in Form eines Fressfeindes erkannt, werden Verhaltensweisen zur Abwehr gezeigt, die beispielsweise eine Detektion durch den Fressfeind minimieren sollen. In dieser post-Begegnungsphase [P*ost-encounter Phase*] besteht noch kein direkter Kontakt, die Bedrohung ist jedoch vom Möglichen in den Bereich des Tatsächlichen übergegangen.

4 Kommt es letztendlich zum (physischen) Kontakt, wird das Verteidigungs- und Angriffsverhalten [*Circa Strike Behavior*] vorbereitet, um Flucht zu ermöglichen.

Abbildung 3. *Predatory Imminence Continuum* nach Fanselow (1988)



Die prä-Begegnungsphase, in der die Wahrscheinlichkeit zum Kontakt mit Fressfeinden erhöht ist, wird von Fanselow mit Angst assoziiert; Angst in ungewissen Situationen, die eine potenzielle Gefahr darstellen und je nach Beschaffenheit das (Explorations-)Verhalten beeinflussen können. Tests, die angeborene Angstmaße erfassen wollen, machen sich diese Ungewissheit zunutze: Sowohl das Offenfeld als auch das EPM beinhalten ambivalente Anreize, die sowohl Explorations- als auch Vermeidungstendenzen evozieren können, da sie die Anfälligkeit für potenzielle Schäden erhöhen. Zusätzlich zu den funktionalen Verhaltensweisen dieser Phase postuliert Fanselow neuronale Korrelate, die für die prä-Begegnungsphase spezifisch sind. Sowohl der Nucleus striae terminalis [*Bed Nucleus of the Stria Termalis;* BNST] und der präfrontale Kortex [*Prefrontal Cortex;* PFC] seien für die Integration situationsspezifischer Informationen entscheidend und grenzten sich dadurch von den neuronalen Korrelaten des Furchtverhaltens ab (Fanselow und Ponnusamy 2008; Perusini und Fanselow 2015). Die post-Begegnungsphase, in der eine reale Gefahr von dem tatsächlichen Vorhandensein einer Bedrohung ausgeht, assoziiert Fanselow mit Furcht, auf die mit speziesspezifischen Verhaltensweisen reagiert werde. Im Falle von Ratten sei dies beispielsweise *Freezing*, da es die Wahrscheinlichkeit einer Detektion durch den Fressfeind minimiere. Furcht sei dabei charakteristisch mit den Hirnregionen Amygdala und PAG assoziiert (Fanselow und Ponnusamy 2008; Perusini und Fanselow 2015).

1.2.2 Erhöhtes Plus-Labyrinth

Das erhöhte Plus-Labyrinth [*elevated plus maze; EPM*] ist das am häufigsten verwendete Tiermodell in der präklinischen Forschung zu Angstzuständen und eignet sich als Modell unkonditionierter Reaktionen auf moderat bedrohliche Situationen (Litvin et al. 2008). Nager und andere Beutetiere befinden sich in neuen, unbekannten Umgebungen in einem Annäherungs-Vermeidungskonflikt, der im EPM als angstähnliches Verhalten quantifiziert werden kann (Montgomery 1955). Die Unterteilung des EPM in offene und geschlossene Bereiche soll diesen Konflikt im Tier abbilden und diente in erster Linie zur Validierung angstlösender Substanzen (Pellow et al. 1985). Die geschlossenen Bereiche stellen dabei eine erhöhte Sicherheit gegenüber Fressfeinden dar und sind somit ein Modell der räuberischen Interaktion mit geringerer Intensität als die reale Konfrontation mit dem Raubtier (Dawson und Tricklebank 1995). Das Vermeiden der offenen Bereiche und Verhaltensweisen zur Gefahrenabschätzung [*Risk Assessment]*, bei dem der Körper des Tieres in den geschützten Bereichen verbleibt und die offenen Bereiche lediglich mit dem Kopf exploriert werden, quantifizieren angstähnliches Verhalten (Cruz et al. 1994). Tiere mit einem erhöhten Angstlevel vermeiden dementsprechend die ungeschützten Bereiche und zeigen

mehr Verhalten zur Gefahrenabschätzung (für eine Übersicht: Litvin et al. 2008). Substanzen mit anxiolytischer Wirkung hingegen verringern das Verhalten zu Gefahrenabschätzung (Pawlak et al. 2008). Vor allem Benzodiazepine – eine der gängigsten Stoffgruppen in der humanen Behandlung pathologischer Angstzustände – und GABA-/glutamaterg wirkende Manipulationen lösen zuverlässig Verhaltensänderungen im EPM aus (Carobrez und Bertoglio 2005). Andere angstregulierende Substanzen, wie beispielsweise Serotoninrezeptor Agonisten, zeigen hingegen unterschiedliche Wirkungen je nach Untersuchung (Übersicht: Lister 1990). Im Vergleich zu den umfassend untersuchten Effekten verschiedener Substanzen auf lokomotionsbasiertes Verhalten, gibt es verhältnismäßig wenig Berichte über USV in Verbindung mit dem EPM. Im Zusammenhang mit den angstevozierenden Effekten der Konfrontation mit dem EPM wird ein Rückgang der 50-kHz USV (Rao und Sadananda 2015; Wöhr et al. 2008) oder sogar ein vollständiges Ausbleiben jeglicher USV (Borta et al. 2006) verzeichnet.

1.2.3 Furchtkonditionierung

Der Furchterwerb ist ein adaptiver Prozess, der für das Überleben in dynamischen Umgebungen entscheidend ist (Lonsdorf et al. 2017). Die Pawlow'sche Furchtkonditionierung ist ein Prozess, der normalerweise eine adaptive Rolle bei der Erzeugung von Verteidigungsverhalten in bedrohlichen Situationen spielt und dazu führt, dass neutrale Reize, die an eine bedrohliche Situation erinnern, Furchtreaktionen in nicht bedrohlichen Situationen auslösen können (Fanselow und Ponnusamy 2008). Das Modell der Furchtkonditionierung ist hierbei von hervorragendem translationalem Wert, sowohl von Nagetieren auf den Menschen und damit verbundene klinische Populationen und zurück (Lonsdorf et al. 2017). Die Furchtkonditionierung ist dabei das „übergreifende exprimentelle Verfahren, das alle Phasen eines Konditionierungsexperiments umfasst", zu dem folgende Bestandteile gehören (nach Lonsdorf 2017):

1) Furchtakquisition – der Prozess des Furchtlernens. Der Erwerb konditionierter Furcht wird durch die Präsentation eines Reizes (konditionierter Reiz; *conditioned Stimulus*; CS) in Verbindung mit einem aversiven Ereignis (unkonditionierter Reiz; *unconditioned Stimulus*; US) erreicht. Infolge dieser Kopplung findet Furchtlernen statt, das sich in der Entwicklung konditionierter Reaktionen (*conditioned Reaction*, CR) auf den CS manifestiert. Im Falle tierexperiementeller Untersuchungen besteht der CS meist aus Ton-, Licht- oder Umgebungsreizen, die mit elektrischer Stimulation oder einem Luftstoß (US) gepaart werden.

Furchtindikatoren (CR) können beispielsweise Schreckreaktionen, Verhaltensstarre (Immobilität, *Freezing*) oder 22-kHz USV sein.

2) Furchtextinktion – der Prozess des Rückgangs der Häufigkeit und/oder des Ausmaßes der konditionierten Reaktion als Folge des Extinktionstrainings. Durch die wiederholte Exposition gegenüber dem CS ohne US kommt es zu einer Abnahme der konditionierten Reaktion, und ist somit ein Indikator erfolgreicher Löschung/Extinktion. In tierexperimentellen Untersuchungen beispielsweise werden nach wiederholter Darbietung eines Tones in der Extinktionsphase weniger oder keinerlei konditionierte Reaktionen in Form von Immobilität gemessen.

3) spontane Erholung [*Recovery*] – bezeichnet die Rückkehr der konditionierten Reaktion in Abhängigkeit der Zeit trotz erfolgreichem Extinktionslernens und weist somit ein Defizit in der Erinnerung an die Extinktion [*Extinction Recall*] durch die Wiedererlangung der zuvor gelöschten konditionierten Reaktion aus. In tierexperimentellen Untersuchungen beispielsweise zeigt sich Recovery in erhöhten Immobilitätsleveln des *Extinction Recall*, wenn diese mit den vorhergehenden Immobilitätsleveln am Ende der Extinktionsphase verglichen werden.

Immobilität [*Freezing*] wird standardmäßig als Maßstab konditionierter Furcht verwendet (Fanselow und Gale 2003). Die furchtinduziert Immobilität nimmt mit steigender Durchgangszahl und Intensität der elektrischen Stimulation zu (Wöhr et al. 2005; Fanselow und Bolles 1979). Daher wird angenommen wird, dass dieses Verhaltensmaß sensitiv gegenüber parametrischen Manipulationen ist, die das Level des Lernens und der daraus resultierenden Furcht beeinflussen (Fanselow und Ponnusamy 2008). Ein positiver Zusammenhang zwischen Intensität der elektrischen Stimulation und USV-Emission wurde auch für 22-kHz USV gefunden (Wöhr et al. 2005).

1.2.4 Raubtiergeruch

Unter kontrollierten Bedingungen zeigen Nagetiere, die dem Geruch von Raubtieren ausgesetzt sind, eine Abnahme des Explorationsverhaltens in Verbindung mit einer Zunahme von *Freezing*, Vermeidungsverhalten und Gefahrenabschätzung (Fanselow und Lester 1988; Takahashi et al. 2008; Apfelbach et al. 2005). Neben der Beobachtung unkonditionierter Reaktionen auf die Konfrontation mit Raubtiergeruch, werden lernabhängige Modelle der Vermeidung von Raubtiergeruch genutzt, um beispielsweise lernabhängige Neuroadaptionen in Angstmodellen zu untersuchen (Übersicht: Staples 2010). Die Vermeidung des Raubtiergeruchs wird darüber hinaus

als Tiermodell für PTBS diskutiert, da sich hier interindividuell unterschiedlich starke Vermeidungsreaktionen auf aversive Stimuli nachweisen lassen, die ebenso wie im Humanbereich mit einer Aktivierung der Hypothalamus-Hypophysen-Nebennierenrinden-Achse (HPA-Achse) assoziiert sind (Albrechet-Souza und Gilpin 2019).

Für eine bessere Vergleichbarkeit zwischen Untersuchungen wird stets angestrebt, standardisierbare Stimuli zu verwenden, und neben natürlichen (in Anteilen schwankenden) Raubtiergerüchen artifizielle Derivate dessen zu nutzen. 2,5-Dihydro-2,4,5-Trimethylthiazolin (TMT), eine synthetische Verbindung, die aus Fuchskot isoliert wurde (Vernet-Maury 1980), besticht hierbei durch ähnliche neuronale und behaviorale Verhaltensänderungen wie andere Raubtiergerüche, da sie ähnliche furchtsame Reaktionen hervorruft (Übersicht: Fendt et al. 2005). Allerdings ist weiterhin fraglich, ob das überlappende Verhaltensprofil auf der Nähe zu Raubtiergeruch fußt oder ob es sich hierbei lediglich um einen prinzipiell als schädlich [*noxious*] wahrgenommenen Geruch handelt (Fendt et al. 2005).

Trotz ihrer hohen Relevanz im natürlichen Umfeld existieren – im Vergleich zu den lokomotionsbasierten Verhaltensweisen – nur sehr wenige Berichte über USV, die durch den Kontakt mit Raubtieren oder Raubtiergeruch ausgelöst werden. Blanchard et al. (1990, 1991b, 1992) zeigten, dass die Konfrontation von Ratten mit einer Katze erfolgreich die Emission 22-kHz USV auslösen. In Studie zu selektiv gezüchteten Tieren, die eine hohe oder niedrige Anzahl an 50-kHz USV produzieren, zeigen sich unterschiedliche Effekte der Exposition gegenüber eines getragenen Katzenhalsbandes. Selektiv häufig 50-kHz USV vokalisierende Tiere zeigen hier eine niedrige Rate geruchsinduzierten 22-kHz USV, selektiv wenig 50-kHz USV vokalisierende Tiere keinerlei geruchsinduzierte 22-kHz USV. Die Kontrolllinie produzierte jedoch eine beträchtliche Anzahl an 22-kHz USV (Webber et al. 2012).

1.2.5 Anxiogene Wirkstoffe

Neben externen Auslösern wie neuen, unbekannten Umgebungen, Raubtiergeruch oder Konfrontation mit elektrischer Stimulation, können auch pharmakologische Manipulationen angstähnliche Zustände hervorrufen. Die fünf Medikamente, die zu diesem Zweck in Studie I ausgewählt wurden, waren Yohimbin, FG 7142, Pentylenetetrazol (PTZ), Meta-Chlorphenylpiperazin (mCPP) und Koffein. Alle verwendeten Substanzen können systemisch verabreicht angstinduzierend wirken – sowohl bei Mensch und Ratte (siehe Tabelle 1). Die Befundlage zur USV-Produktion der Ratte unter diesem Einfluss ist bislang wenig untersucht.

Tabelle 1. Publizierte Effekte anxiogener Substanzen.

	Freezing und Immobilität	*EPM (Dauer offene Arme)*	*USV*	*Lokomotorische Aktivität*	*Angstauslösend (Mensch)*
Yohimbin	↑ Bhattacharya et al. 1997 Park et al. 2001	↓ Baldwin und File 1989 Baldwin et al. 1989 Bhattacharya et al. 1997 Cole et al. 1995 Gill et al. 2013 Khoshbouei et al. 2002 Pellow et al. 1985	22 ↓ Molewijk et al. 1995a Vry et al. 1993b 40 / Krall et al. 2005	/ Chen et al. 2015 ↓ Bhattacharya et al. 1997 Pellow et al. 1985	ja Charney et al. 1983 Southwick et al. 1993
FG 7142	↑ Beck und Cooper 1986	↓ Cole et al. 1995 Cruz et al. 1994 Pellow und File 1986	22 ± Jelen et al. 2003 22 / Kaltwasser 1991 40 / Olivier et al. 1998 40 ↑ Gardner und Budhram 1987		ja Dorow et al. 1983
PTZ	n/a	↓ Cole et al. 1995 Cruz et al. 1994 Garcia et al. 2011 File und Pellow 1985 Ramos et al. 2008 Wada und Fukuda 1991 Wallis und Lal 1998	40 ↑ Carden et al. 1993	↓ Pellow et al. 1985 Ramos et al. 2008	ja Good 1940 Rodin 1958
mCPP	n/a	↓ Buczek et al. 1994 Gibson et al. 1994 Shepherd et al. 1994 Wallis und Lal 1998	22 ↓ Vry et al. 1993b (e.Stim) 22 ↑ Campbell und Merchant 2003 (Offenfeld) 40 ↓ Winslow und Insel 1991	/ Lucki et al. 1989 Kennett und Curzon 1988	ja Murphy et al. 1989 Charney et al. 1987
Koffein	Bhattacharya et al. 1997 Antoniou et al. 1998	↓ Baldwin et al. 1989 Bhattacharya et al. 1997 Pellow et al. 1985 ↑ Garcia et al. 2011	50 Simola et al. 2010 (qualitativ)	↑ Antoniou et al. 1998 Bennett und Semba 1998 Garrett und Holtzman 1994 ↓ Bhattacharya et al. 1997	ja Foltin 1991

Anmerkung. Abgebildet sind Stimulierende (↑) und hemmende (↓) Effekte verschiedener Substanzen. n/a – keine Information. EPM – erhöhtes Plus-Labyrinth, angstähnliches Verhalten wird durch verminderte Verweildauer in den offenen Armen (↓) indiziert. USV – Ultraschallvokalisationen: 22 – 22-kHz USV, 40 – isolations-induzierte Vokalisation von Jungtieren, 50 – 50-kHz USV. / - keine Effekte, ± - gemischte Effekte

1.3 Biologische Faktoren

Abgesehen von den systematischen Einflüssen situationsspezifischer Faktoren auf Angst und Furcht gibt es eine große interindividuelle Variabilität in humanen Populationen und stabile Merkmale scheinen eine wichtige modulierende Rolle für den Erfolg therapeutischer Maßnahmen zu spielen (Holmes und Singewald 2013). Neben externen Einflüssen können interindividuelle Unterschiede auf die systematische Variation biologischer Komponenten zurückzuführen sein. Allen voran sollen hier sowohl geschlechtsspezifische Effekte, als auch Unterschiede der serotonergen Transmission in Bezug auf Angst und Furcht thematisiert werden. Ebenso kann der Einfluss stabiler Persönlichkeitseigenschaften [*Traits*] berücksichtigt werden.

1.3.1 Geschlecht

Obwohl Frauen doppelt so häufig an Angststörungen erkranken wie Männer (Altemus et al. 2014; Holden 2005; Kessler et al. 1994; Kirkpatrick et al. 2013; Seeman 1997) und gleichzeitig schwerere Symptome zeigen (Seedat et al. 2005), wird dieser Geschlechtsdimorphismus in der präklinischen Forschung beispielsweise zur Furchtkonditionierung bei Nagetieren und Menschen häufig vernachlässigt (Lonsdorf und Merz 2017). Es gibt Hinweise darauf, dass die konditionierte Furchtreaktion bei männlichen Ratten größer ausfällt als bei ihren weiblichen Artgenossen, während Weibchen ein höheres Maß an unkonditionierter Furcht zeigen (Kosten et al. 2005). Die Befunde zu geschlechtsabhängigen USV sind gemischt. Seminaturalistische Untersuchungen deuten auf eine höhere Defensivität weiblicher Tiere hin (Blanchard et al. 1991a; Blanchard et al. 1992; Shepherd et al. 1992), während die Konfrontation mit einem Luftstoß (Inagaki und Mori 2014; Inagaki und Sato 2016) oder elektrischer Stimulation (Graham et al. 2009; Kosten et al. 2005; Kosten et al. 2006; Vry et al. 1993b) mit systematisch weniger 22-kHz USV der Weibchen einhergeht. Werden die Maße angstähnlichen Verhaltens in verschiedenen Tests in Abhängigkeit des Geschlechts aggregiert, zeigt sich wiederum – entgegen der klinischen Realität im Humanbereich – ein weniger ängstliches Verhaltensprofil bei weiblichen Ratten (Aguilar et al. 2003; Lopez-Aumatell et al. 2008). Die Untersuchung beider Geschlechter im Tiermodell ist demnach unerlässlich, um die geschlechtsabhängigen Effekte auf Angst und Furcht berücksichtigen zu können und deren Verhältnis zur Humanpopulation zu analysieren.

1.3.2 Serotonin

Serotonin (5-HT) hat einen weitreichenden Einfluss auf diverse regulatorische Prozesse und modelliert unterschiedlichste Verhaltensweisen. Sowohl die Hirnentwicklung als auch die neuronale Plastizität im Erwachsenenalter werden von 5-HT beeinflusst (Whitaker-Azmitia 2010). Als phylogenetisch sehr altes Neurotransmittersystem haben 5-HTerge Neurone ihren Ursprung in den Raphé-Kernen [D*orsal Raphé Nucelus*, DRN] und innervieren das Rückenmark und den Hirnstamm (Dahlström und Fuxe 1964). Durch absteigende Projektionen über die Medulla beeinflusst 5-HT verschiedene physiologische Systeme und basale Funktionen wie Atmung, Thermoregulation oder den Schlaf-Wachzyklus und diverse sensomotorische Funktionen (Carey 2010). Aber auch komplexere Körperfunktionen wie Appetit und Sättigungsgefühl (Lee und Clifton 2010) sowie das Sexualverhalten (Uphouse und Guptarak 2010) sind mit dem 5-HTergen System assoziiert. Zudem innervieren 5-HTerge Fasern nahezu alle kortikalen und subkortikalen Bereiche des Gehirns, wie beispielsweise den präfrontalen Kortex oder das limbische System (Hornung 2010) und sind hier meist durch inhibierende Prozesse an der Regulation von Verhaltensweisen, wie beispielsweise der Hemmung lokomotorischer Aktivität, beteiligt (Lucki 1998).

Die emotionale Regulation wird ebenso maßgeblich von 5-HTerger Transmission gesteuert und kann durch pharmakologische Manipulationen verändert werden (Hensler 2010). Allen voran ist 5-HT an der Regulation von Angst und Furcht beteiligt (Bauer 2015; Deakin und Graeff 1991; Lowry und Hale 2010) und steht beispielsweise in engem Zusammenhang mit Angststörungen, einschließlich PTBS (Guimarães et al. 2010; Gordon und Hen 2004). Zum einen begünstigen die 5-HTergen Projektionen des DRN in die Amygdala und den präfrontalen Kortex die Furchtkonditionierung, zum anderen hemmen die Projektionen ins PAG den Ausdruck angeborener Furcht (Deakin und Graeff 1991). Auch die 5-HT-Konzentrationen verschiedener Hirnareale haben verschiedene Auswirkungen auf das Angstverhalten (Guimarães et al. 2010). So ist beispielsweise in der Ratte bei Konfrontation mit einem angstauslösenden Stimulus eine erhöhte, extrazelluläre 5-HT-Konzentration im ventralen Hippocampus und der basolateralen Amygdala, beides Teile des limbischen Systems, nachweisbar (Amat et al. 1998). Abhängig von Wirkort und den involvierten Rezeptoren kann die 5-HTerge Neurotransmission die Angstreaktion dementsprechend verstärken oder abschwächen.

Im 5-HTergen System gibt es sieben verschiedene Rezeptorklassen (5-HT_1 – 5-HT_7) mit jeweils unterschiedlich vielen Subtypen (z.B. 5-HT_{1A}, 5-HT_{2C}; für eine Übersicht der insgesamt 14 Subtypen: Andrade et al. 2019; Hannon und Hoyer 2008; Hoyer et al. 2002), denen jeweils

spezifische Effekte zugewiesen werden. So hat im Tiermodell beispielsweise die Gabe von 5-HT_{2C}-Rezeptor-Agonisten einen anxiogenen Effekt (Campbell und Merchant 2003), während der somatodendritische 5-HT_{1A}-Autorezeptor neben der Regulation der synaptischen Übertragung innerhalb der hypothalamischen und autonomen Zentren bei Stimulation durch einen Agonisten angstlösend wirkt (Vry 1995; Prut und Belzung 2003). Die 22-kHz USV-Emission wird hierbei als Marker genutzt, um die Wirksamkeit anxiolytischer Wirkstoffe zu untersuchen, von denen die meisten das serotonerge System ansteuern. Pharmakologische Studien zur 22-kHz USV-Emission konzentrieren sich vor allem auf die 5-HT_1- und in geringerem Maße auch die 5-HT_2-Rezeptorfamilien (Baudrie et al. 1993; Molewijk et al. 1995b; Sánchez 1993; Schreiber et al. 1998; Schreiber und Vry 1993; Sommermeyer et al. 1993; Vry et al. 1993a; Vry et al. 2004). Vollständige oder partielle 5-HT_{1A}-Rezeptor-Agonisten, einschließlich 8-OH-DPAT, Buspiron, Gepiron und Ipsapiron, blockieren hierbei die Emission von 22-kHz USV (Sánchez 2003b; Wöhr et al. 2015; Wöhr und van Gaalen 2018). Ein weiteres primäres Ziel pharmakologischer Studien ist der Serotonintransporter (SERT) und eine umfangreiche Reihe von Experimenten zeigte, dass selektive Serotonin-Wiederaufnahmehemmer (SSRIs) die 22-kHz USV-Emission hemmen (Kassai und Gyertyán 2018; Sánchez 2003a).

1.3.2.1 SERT-KO

Ein wichtiger Bestandteil 5-HTerger Übertragung ist, neben der Vielzahl an unterschiedlichen Rezeptoren, der Serotonintransporter (5-HTT; SERT; SLC6A4), der die Verfügbarkeit von 5-HT im synaptischen Spalt reguliert und die 5-HT-Signalübertragung durch Wiederaufnahme von 5-HT in die präsynaptische Endigung beendet (Murphy und Lesch 2008). Ist die Verfügbarkeit des SERT gestört, kann es zu neuronalen und behavioralen Veränderungen kommen. Die polymorphe Region im Promotor des SERT-Gens (5-HTTLPR) führt beim Menschen zur Bildung von kurzen (s) und langen (l) allelischen Varianten, wobei die kurze Variante mit einer reduzierten Transkription und einer veränderten Funktion von SERT assoziiert wurde (Canli und Lesch 2007). Es gibt Hinweise, dass die genetische Variation bei Merkmalsträgern eines oder zwei kurzer Allele (s/l oder s/s) geht mit einer veränderten Amygdala-Aktivierbarkeit als Antwort auf negative Stimuli (Hariri et al. 2002) einhergeht und zu einer verringerten Stabilität inhibitorischen Lernens führt (Wannemüller et al. 2018). Früheren Untersuchungen zufolge führt diese genetische Disposition zu einer erhöhten Vulnerabilität für Depressionen (Dorado et al. 2007), Angsterkrankungen (Furmark et al. 2004) und der Ausprägung ängstlicher Charaktereigenschaften (Lesch et al. 1996). Verschiedene Studien postulieren, dass die

Interaktion mit stressvollen Lebenssituationen oder traumatischen Ereignissen Personen mit einem oder zwei kurzen Allelen eher depressive Symptome und diagnostizierbare Depressionen (Caspi et al. 2003) oder eine PTBS ausbilden (Gressier et al. 2013). Neueste Meta-Analysen zeigen jedoch, dass dieser Zusammenhang lediglich schwach, nur unter spezifischen Voraussetzungen (Risch et al. 2009) oder bisweilen überhaupt nicht gegeben ist (Culverhouse et al. 2018).

Um eine veränderte synaptische Übertragung auf Grund einer Veränderung des zur Verfügung stehenden Transporters im Tiermodell nachstellen zu können, bedient man sich genetisch veränderten Modellen, die die Verfügbarkeit des SERT beeinflussen (Bengel et al. 1998; Holmes et al. 2003; Homberg et al. 2007a). Eine vermehrte Expression des SERT geht beispielsweise mit einem geringeren, angstähnlichen Verhalten einher (Jennings et al. 2006). Das komplette Ausschalten des SLC6A4 (SERT-„Knockout“, SERT-KO) durch genetische Manipulation hingegen führt in den Hippocampi von Mäusen und Ratten zu einer sechs- bis neunfach erhöhten Konzentration extrazellulären 5-HTs (Fabre et al. 2000; Homberg et al. 2007a). Die fehlende Wiederaufnahme führt jedoch prinzipiell zu einem niedrigeren 5-HT-Spiegel im Neuron selbst und weniger Depolarisations-induzierter Ausschüttung (Homberg et al. 2007a). Ratten mit permanent erhöhtem 5-HT-Spiegel und gleichermaßen weniger effizienter Transmission zeigen verstärktes angstbezogenes Verhalten. Zum einen zeigt sich ein anxiogener Phänotyp in unkonditionierten Aufgaben wie dem Offenfeld, dem EPM, dem Hell-Dunkel-Test und der durch Neuheit unterdrückten Nahrungsaufnahme (Golebiowska et al. 2019; Johnson et al. 2019; Olivier et al. 2008; Schipper et al. 2011). Zum anderen wird die Löschung konditionierten Furchtverhaltens durch die Verfügbarkeit von SERT modelliert (Homberg 2012). Während die Furchtakquisition von Bedrohungssignalen in den meisten Studien nicht beeinträchtigt scheint, wurde die Fähigkeit, konditioniertes Verhalten zu extingieren, wiederholt als gestört beobachtet (Luoni et al. 2013; Nonkes et al. 2012; Schipper et al. 2011; Schipper et al. 2018; Schipper et al. 2019a; Schipper et al. 2019b; Shan et al. 2014; Shan et al. 2018). Darüber hinaus behindert das Fehlen von SERT den Extinktionsabruf, was sich in einer Recovery der zuvor gelöschten konditionierten Immobilität zeigt (Schipper et al. 2019b; Schipper et al. 2018). Obwohl die Regulation der 22-kHz USV-Emission maßgeblich von 5-HT beeinflusst ist und SERT eine wichtige Rolle bei Angst und Furcht spielt, ist bisher nicht bekannt, wie die SERT-Verfügbarkeit die Emission von 22-kHz USV unter angstähnlichen und furchtsamen Manipulationen bei Ratten beeinflusst.

1.3.3 *Trait Anxiety* – Ängstlichkeit

Angst kann in eine Zustands- und eine Merkmalsdimension unterteilt werden, welche den situationsbedingten, akuten Zustand der Angst [S*tate Anxiety*] von der generellen Angstneigung [T*rait Anxiety*] unterscheidet (Lonsdorf und Merz 2017; Spielberger 1983). Die *State Anxiety* bezieht sich darauf, wie ängstlich eine Person im Moment ist (intraindividuelle Unterschiede) und umfasst somit situationsspezifische Änderungen des Angstniveaus; wohingegen sich *Trait Anxiety* auf die Ängstlichkeit einer Person im Allgemeinen bezieht (interindividuelle Unterschiede, nach Lonsdorf und Merz 2017). Es wird angenommen, dass *Trait Anxiety* möglicherweise Prozesse der Furchtkonditionierung vorhersagen, jedoch ist die Befundlage im Humanbereich zum Einfluss der allgemeinen Angstneigung auf die Performanz während der Furchtkonditionierung gemischt (Übersicht: Lonsdorf und Merz 2017).

Im Tiermodell werden für den zu untersuchenden *Trait* charakteristische Verhaltensweisen definiert, die Tiere entsprechend ihrer Performanz in hohe vs. niedrige Ausprägung unterteilt und in Abhängigkeit dessen auf systematische Variation in weiterführenden Tests untersucht. So gelten Ratten mit einer langen Verweildauer in den offenen Bereichen des EPM als weniger ängstlich (HOA, *high open arm*) als Ratten, die dort weniger Zeit verbringen (LOA, *low open arm*; Borta et al. 2006). Untersuchungen zeigen, dass ängstliche Ratten ein höheres Maß an furchtinduzierter Immobilität zeigen (Borta et al. 2006; Ilse et al. 2019), angstrelevante Hinweise besser diskriminieren können (Kreutzmann et al. 2021); jedoch ein langsameres, aktives Vermeidungslernen zeigen (Ho et al. 2002). Ängstlichkeit bei Mäusen (Sartori et al. 2011) und Ratten (Fendt et al. 2021) sagt zudem ein verstärktes Furchtgedächtnis nach Furchtkonditionierung vorher. Zudem extingieren Mäuse mit hoher Ängstlichkeit langsamer und weniger erfolgreich (Godoy et al. 2022). Defizite bei der Extinktion und dem Extinktionsabruf finden sich auch bei Ratten, die selektiv auf hohe Ängstlichkeit gezüchtet wurden (Muigg et al. 2008). In diesem Zusammenhang wurde festgestellt, dass interindividuelle Unterschiede bei der Extinktion zu systematischen Unterschieden bei der Recovery führen, wobei langsam extingierende Ratten anfälliger für einen Rückfall der Angst sind als ihre schnell extingierenden Artgenossen (King et al. 2018).

Neben den lokomotorisch basierten Maßen hängt auch die USV-Produktion von der Angstneigung der Ratten ab. Ängstliche Ratten zeigen hierbei verstärkte 22-kHz USV-Emission während der Furchtkonditionierung (Borta et al. 2006) und 50-kHz USV während sozialer Situationen (Lukas und Wöhr 2015). Ähnliche Befunde berichten Untersuchungen zum Tiermodell der depressionsähnlichen Symptomatik (Wistar-Kyoto-Ratten, Rao und Sadananda 2015). Die

Untersuchung von Roa und Sadananda (2015) wies keine angstinduzierte Reduktion der 50-kHz USV im Plus-Labyrinth, jedoch eine verringerte sozial induzierte 50-kHz USV-Produktion der Wistar-Kyoto-Ratten nach. Auch andere Modelle zeigten eine Reduktion der Tickling-induzierten 50-kHz USV bei Ratten mit ängstlich-depressivem Phänotyp (Turner et al. 2019). Andererseits kann auch die Produktion von USV als Prädiktor interindividueller Unterschiede im Angstverhalten dienen. Die unterschiedlich stark ausgeprägte Reaktivität auf Tickling mit 50-kHz USV hängt mit Ängstlichkeit der Ratten im EPM zusammen (Mällo et al. 2007b) – und hat in Abhängigkeit des Geschlechts unterschiedliche Effekte auf die Stressreaktion (Mällo et al. 2009).

2 FRAGESTELLUNG UND HYPOTHESEN

Nach Fanselow (1988) unterscheiden sich Angst und Furcht durch divergierende Verhaltenskorrelate. Da sowohl Angstverhalten, beispielsweise im EPM, als auch Furcht, in Paradigmen der Furchtkonditionierung, mit einer generellen lokomotorischen Inhibition einhergehen, wird eine Differenzierung beider Phänomene mittels der gemessenen USV angestrebt. Die bisherige Zuordnung der emittierten USV zu negativen affektiven Zustände wie Angst und Furcht ist jedoch nicht konsistent. Die Emission von 22-kHz USV soll hierbei entweder einen „negativen affektiven Zustand widerspiegeln, der Angst und Furcht ähnelt" (Wöhr und Schwarting 2013; Willadsen et al. 2021b) oder wird explizit mit Angst und nicht mit Furcht in Verbindung gebracht (Brudzynski 2021; Jelen et al. 2003). 50-kHz USV wiederum werden weitaus weniger oft mit negativen affektiven Zuständen assoziiert (Brudzynski 2021), obgleich sie in agonistischen Interaktionen (Vivian und Miczek 1993) oder in potenziell gefährlichen, nicht-belohnenden und nicht-sozialen Umgebungen zu finden sind (Engelhardt et al. 2017; Knutson et al. 1999; Pereira et al. 2014; Rippberger et al. 2015; Thompson et al. 2006; Wintink und Brudzynski 2001; Wöhr et al. 2014). Wenn 22-kHz USV einen negativen affektiven Zustand der Angst wiederspiegeln, sollten sie bereits in potenziell gefährlichen Situationen während pharmakologisch induzierter angstähnlicher Zuständen messbar sein (Studie I). Es wurde außerdem erwartet, dass akute Bedrohungsszenarien – wie die Konfrontation mit Raubtiergeruch (Studie II), elektrische Stimulation oder konditionierter Stimuli (Studie III und IV) von 22-kHz USV-Emissionen begleitet werden. Inwiefern die Produktion von 50-kHz USV durch Angst und Furcht beeinflusst wird, bleibt zu klären.

Die Amplitude der gezeigten Abwehrreaktion nimmt hierbei im Sinne des *Predatory Imminence Continuum* mit zunehmender Nähe zur Bedrohung zu (Fanselow und Lester 1988; Perusini und Fanselow 2015; Fanselow und Ponnusamy 2008). Zugleich steht die Emission der 22-kHz USV in einem Dosis-Wirkungs-Zusammenhang mit der Intensität aversiver Situationen (Wöhr et al. 2005). Es wurde erwartet, dass pharmakologisch induzierte, angstähnliche Zustände von einer veränderten USV-Emission begleitet werden (Studie I) und die 22-kHz USV-Emission in Abhängigkeit der Nähe zur Bedrohung in akuten Bedrohungsszenarien (Studie II, III und IV) zunimmt.

Neben der Unterscheidung von Angst und Furcht aufgrund situativer Begebenheiten sollte zudem der Einfluss verschiedener biologischer Faktoren auf das Spektrum der USV untersucht werden. Geschlechtsabhängige Befunde deuten darauf hin, dass weibliche Ratten weniger angstähnliches Verhalten zeigen (Aguilar et al. 2003; Lopez-Aumatell et al. 2008) und in Furchtkonditionierungsparadigmen weniger 22-kHz USV emittieren (Graham et al. 2009; Kosten et al. 2006; Kosten et al. 2005; Vry et al. 1993b). Demnach wurde erwartet, dass weibliche Ratten

eine geringeres angstähnliches Verhalten im EPM zeigen (Studie IV) und weniger furchtinduzierte 22-kHz USV während der Furchtkonditionierung produzieren (Studie III und IV).

Obgleich die veränderte serotonerge Transmission des SERT-KO-Modells bereits häufig mit Defiziten im Bereich der Furchtkonditionierung in Verbindung gebracht wurde (Luoni et al. 2013; Nonkes et al. 2012; Schipper et al. 2011; Schipper et al. 2018; Schipper et al. 2019a; Schipper et al. 2019b; Shan et al. 2014; Shan et al. 2018) , ist wenig bekannt darüber, wie die SERT-Verfügbarkeit die Emission von 22-kHz USV in angst- oder furchtähnlichen Zuständen bei Ratten beeinflusst. Ausgehend von dem Einfluss gestörter serotonerger Transmission ist von den SERT-KO-Ratten mehr angstähnliches Verhalten zu erwarten, das sich ebenso in einer veränderten USV-Produktion niederschlagen sollte (Studie III und IV).

Ein weiterer Prädiktor für die Performanz während des Furchtkonditionierungsparadigmas ist die Ausprägung der Ängstlichkeit. Erhöhte Ängstlichkeit wird mit Defiziten in der Furchtkonditionierung (Borta et al. 2006; Fendt et al. 2021; Ilse et al. 2019; Muigg et al. 2008) und vermehrter 22-kHz USV-Produktion assoziiert (Borta et al. 2006; Fendt et al. 2021). Es wurde demnach erwartet, dass Ratten, die weniger Zeit in den offenen Armen des EPM verbringen, höhere Furchtlevel und eine vermehrte 22-kHz USV-Produktion während des Furchtkonditionierungsparadigmas zeigen (Studie IV).

3 VORGELEGTE ARBEITEN - ZUSAMMENFASSUNGEN

STUDIE I – WILLADSEN ET AL. 2018

Willadsen M, Best LM, Wöhr M, & Clarke PBS (2018). Effects of anxiogenic drugs on the emission of 22-and 50-kHz ultrasonic vocalizations in adult rats. *Psychopharmacology*, 235(8), 2435-2445.

Ultraschallvokalisationen von Ratten enthalten Informationen über deren affektiven Zustand. Während aversive Situationen in adulten Ratten und Jungtieren hauptsächlich mit Vokalisationen im Frequenzbereich um die 22 kHz, beziehungsweise 40 kHz, einhergehen, werden appetitive Situationen mit Rufen im Bereich um 50 kHz assoziiert. Die Klasse der 50-kHz USV setzt sich aus einer Vielzahl einzelner Ruftypen zusammen, die je nach Manipulation verschieden stark vertreten sein können und deren unterschiedliche Bedeutung weithin diskutiert wird. So lässt sich beispielsweise beobachten, dass nach Gabe von Amphetamin und anderen belohnenden Stimuli vermehrt komplexe 50-kHz Vokalisationen (*Trills*) das Rufprofil bestimmen. Ob diese und andere Veränderung des Rufprofils auch mit angstähnlichen Zuständen in Verbindung gebracht werden können, gilt es zu untersuchen.

Hierzu wurden in dieser Studie die Verhaltensantworten von männlichen Long-Evans-Ratten auf diverse, bekanntermaßen anxiogene Substanzen (PTZ, mCPP, FG-7142, Yohimbin, Koffein) mit amphetamininduzierten Veränderungen in einem Offenfeld-Paradigma verglichen. Es zeigte sich, dass sowohl Koffein als auch Amphetamin stimulierend auf die lokomotorische Aktivität und 50-kHz USV-Produktion wirken, während PTZ, mCPP und FG-7142 den gegenteiligen Effekt aufweisen und mit einer vermehrten Inaktivität und reduzierter Rufproduktion einhergingen. Letztere Substanzen induzierten überzeugend einen angstähnlichen Zustand, der sich jedoch nicht in einer Veränderung des Rufprofils niederschlug. Somit wurde ausgeschlossen, dass die Veränderung des Rufprofils durch Amphetamin (auch) anxiogene Effekte abbildet.

Die Gabe anxiogener Substanzen und die entsprechende Induktion eines angstähnlichen Zustands war in dieser Studie mit einer Reduktion der positiv konnotierten 50-kHz USV assoziiert. Angstähnliche Zustände ließen dementsprechend nicht im Bereich der 22-kHz USV auf dem Spektrum der situationsspezifischen USV verorten, sondern wurden von einer Reduktion der 50-kHz USV begleitet.

STUDIE II – FENDT ET AL. 2018

Fendt M, Brosch M, Wernecke KE, **Willadsen M**, & Wöhr M (2018). Predator odour but not TMT induces 22-kHz ultrasonic vocalizations in rats that lead to defensive behaviours in conspecifics upon replay. *Scientific reports*, 8(1), 1-11.

Ratten verfügen über verschiedene Strategien, sich vor Fressfeinden zu schützen und Artgenossen vor drohenden Gefahren zu warnen. In Abhängigkeit der Nähe der drohenden Gefahr stellen sie beispielsweise jegliche Bewegung ein (*Freezing*) oder vokalisieren im Ultraschallbereich (USV). Ein zuverlässiger Auslöser defensiven Verhaltens ist hierbei der Geruch eines Fressfeindes – natürlicher oder synthetischer Art. Die akustische Reaktion der Ratte auf diesen Stimulus wurde bisher allerdings nur spärlich untersucht. In der vorliegenden Studie wurden daher männliche Sprague-Dawley-Ratten mit verschiedenen Geruchsproben (Löwen- und Fuchsurin; synthetisches TMT) konfrontiert, wobei deren sichtbares Verhalten, wie *Freezing* oder Abstand zur Probe; sowie ausgestoßene USV gemessen wurden. Hierbei zeigte sich, dass die natürlichen Proben bei ca. 20% der Ratten USV hervorrufen, wohingegen die synthetische Substanz keinerlei akustische Reaktion evoziert. Anders verhält es sich bei dem lokomotorischen Verhalten: Hier sind sowohl die synthetische Substanz als auch der höher konzentrierte Löwenurin in der Lage, Vermeidungsverhalten in der Ratte hervorzurufen.

Die von den Tieren ausgestoßenen Rufe eignen sich wiederum, die Verhaltensantwort eines Empfängertiers im etablierten Playback-Paradigma genauer zu untersuchen. Hier zeigte sich, dass 22-kHz Rufe verschiedenen Ursprungs zu einer Verhaltensinhibition im Empfänger führen. Sowohl von Löwenurin induzierte 22-kHz Rufe als auch Rufe eines furchtkonditionierten Tieres lösten in geringer Lautstärke spezifische Verhaltenseffekte im Playback-Paradigma aus, während vergleichbare Kontrollstimuli dies nicht taten. Die ausgestoßenen Rufe wurden demnach erkannt und übermittelten brauchbare Information. Rückt die Bedrohung durch akustische Signale jedoch näher, in Form von höherer Lautstärke, führten sowohl natürliche 22-kHz USV als auch deren vergleichbare Kontrollstimuli zu einer Verhaltensinhibition.

Demnach evozierten spezifische Stimuli wie der Geruch von Fressfeinden oder elektrische Stimulation in Abhängigkeit ihrer drohenden Gefahr 22-kHz USV, die wiederum als distinktes Abwehrverhalten und Indikator von gerichteter Furcht interpretiert wurden.

STUDIE III – WILLADSEN ET AL. 2021A

Willadsen M, Uengoer, M, Schwarting RKW, Homberg JR, & Wöhr M (2021). Reduced emission of alarm 22-kHz ultrasonic vocalizations during fear conditioning in rats lacking the serotonin transporter. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, 108, 110072.

Ratten verfügen über ein umfangreiches soziales Verhaltensrepertoire. Ein wichtiger Bestandteil dieses Repertoires ist die Aussendung von pfeifenähnliche Rufe im Ultraschallbereich, sogenannten Ultraschallvokalisationen (USV). Lange, niederfrequente 22-kHz USV treten in aversiven Situationen auf, z. B. bei aggressiven Interaktionen, beim Kontakt mit Raubtieren und bei elektrischer Stimulation während der Angstkonditionierung. Es wird angenommen, dass sie einen negativen affektiven Zustand widerspiegeln, der mit Angst und Furcht vergleichbar ist. Serotonin (5-Hydroxytryptamin, 5-HT) spielt eine wichtige Rolle bei der Regulierung von Gefühlszuständen, insbesondere von Angst und Furcht. Eine Schlüsselkomponente des Systems ist der 5-HT-Transporter (5-HTT, SERT), der die Verfügbarkeit von 5-HT im synaptischen Spalt reguliert.

Das vorliegende Experiment untersuchte die Auswirkungen eines SERT-Mangels auf sichtbares, angstbezogenes Verhalten und 22-kHz USV während der Angstkonditionierung bei männlichen und weiblichen Ratten. Während sichtbares, angstbezogenes Verhalten nicht durch SERT-Mangel und Geschlecht beeinflusst wurde, war die Emission von 22-kHz USV bei Serotonintransporter-Knockout (SERT-KO) Ratten im Vergleich zu ihren Wildtyp-Wurfgeschwistern deutlich reduziert. Die Auswirkungen des Genotyps waren bei den Weibchen besonders ausgeprägt. Weibchen stießen im Allgemeinen weniger 22-kHz USV aus als Männchen. Bemerkenswerterweise wurden ebenfalls geschlechtsabhängige Effekte auf die 50-kHz USV-Produktion während der Anfangsphase der Furchtkonditionierung gefunden. Im Gegensatz zu der verringerten 22-kHz USV-Emission zeigten sich hier jedoch verstärkte 50-kHz USV-Emission bei den Weibchen.

Weibliche Ratten behielten demnach in zunehmend aversiver werdenden Situationen die Emission von 50-kHz USV länger bei und vokalisierten gleichsam weniger 22-kHz USV als Männchen in vergleichbaren Situationen. Hierbei schien es sich um eine geschlechtsabhängig Verschiebung auf dem situationsspezifischen Spektrum der USV zu handeln.

STUDIE IV – WILLADSEN ET AL. 2021B

Willadsen M, Uengoer M, Sługocka A, Schwarting RKW, Homberg JR, Wöhr M (2021). Fear Extinction and Predictive Trait-Like Inter-Individual Differences in Rats Lacking the Serotonin Transporter. *International Journal of Molecular Sciences* 22 (13), S. 7088.

Ein charakteristisches Merkmal vieler Angststörungen ist die unzureichende Extinktion von Furchterinnerungen, bei der das serotonerge System (5-Hydroxytryptamin, 5-HT) mit dem 5-HT-Transporter (5-HTT, SERT) spielt hierbei eine entscheidende Rolle spielt. In der vorliegenden Studie wurde die Auswirkungen eines SERT-Mangels auf die Furchtextinktion in einem differentiellen Furchtkonditionierungsparadigma in männlichen und weiblichen Ratten untersucht. Angst- und furchtbezogenes Verhalten während Akquisition, der Extinktion und der spontanen Erholung (*Recovery*) wurde mittels der Quantifizierung von Immobilität und 22-kHz USV gemessen. Eigenschaftsähnliche interindividuelle Unterschiede in Bezug auf Neuheitssuche, angstbezogenes Verhalten, Habituation, kognitive Leistung und Schmerzempfindlichkeit wurden auf ihren prädiktiven Wert für die Vorhersage der Furchtextinktion getestet. Die Ergebnisse legen nahe, dass der SERT-Mangel die Emission von 22-kHz USV während der differenziellen Furchtkonditionierung stark beeinflusst. Während der Akquisition, der Extinktion und der spontanen Erholung, führte SERT-Mangel durchweg zu einer Verringerung der 22-kHz USV-Emission. Während SERT-Mangel die Immobilität während der Akquisition nicht beeinflusste, traten während der Extinktion Unterschiede zwischen den Genotypen auf, und während der Phase der spontanen Erholung zeigten Ratten mit SERT-Mangel ein höheres Maß an Unbeweglichkeit als deren Wildtyp-Wurfgeschwister. Die spontane Erholung spiegelte sich in einer erhöhten Immobilität wider, nicht aber in der 22-kHz USV-Emission. Zudem zeigten sich deutliche Geschlechtsunterschiede. Von den verschiedenen Maßen für eigenschaftsähnliche interindividuelle Unterschiede hatte das angstbezogene Verhalten die beste Vorhersagekraft.

Ergänzende Analysen ergaben, dass neben Geschlecht und veränderter serotonerger Transmission stabile Verhaltensmerkmale wie Ängstlichkeit [*Trait Anxiety*] mit der Performanz während der Furchtkonditionierung assoziiert sind. Die emittierten 50-kHz USV standen hierbei in negativen Verhältnis zu angstähnlichem Verhalten im EPM und in einem positiven Zusammenhang mit der Performanz während Extinktion und Recovery. Wohingegen die 22-kHz USV lediglich mit dem lokomotorischen Verhalten während direkter Konfrontation mit dem US assoziiert sind. Auch hier ließen sich demnach Zusammenhänge zwischen angstähnlichen Verhaltens und 50-kHz USV sowie distinkter Furchtreaktion und 22-kHz USV erkennen.

4 Diskussion

4.1 Einfluss situationsspezifischer Faktoren

4.1.1 50-kHz USV in angstinduzierenden Kontexten

Verschiedene aversive Bedingungen verringern sowohl spontan emittierte 50-kHz USV als auch die durch unterschiedliche Manipulation evozierten 50-kHz USV. Spontan im Offenfeld emittierte 50-kHz USV wurden durch pharmakologisch induzierte angstähnliche Zuständen reduziert (Studie I). Angstähnliche Zustände wurden hierbei über ein gesteigertes Maß an Immobilität definiert, das gleichsam mit einer verringerten 50-kHz USV-Emission einherging. Ähnliche Ergebnisse fanden sich im direkten Vergleich von angstinduzierenden und neutralen Situationen. Die Abnahme der 50-kHz USV im EPM im Vergleich zum Offenfeld-Paradigma deutet ebenfalls darauf hin, dass erhöhte Angstlevel mit einer verringerten 50-kHz USV-Emission einhergehen (Rao und Sadananda 2015; Wöhr et al. 2008). Ebenso wie die angstinduzierte Abnahme führte die Induktion von Stress, beispielsweise durch elektrische Stimulation, zu einer verringerten 50-kHz USV-Emission. Wurden Tiere unmittelbar vor der Testung im Offenfeld mit mehreren Elektroschocks konfrontiert, verringerte sich deren 50-kHz USV-Produktion im Vergleich zu nicht gestressten Individuen (Rojas-Carvajal und Brenes 2020). Auch (sozial) induzierte 50-kHz USV sind sensibel gegen Stress und andere aversive Bedingungen, da durch heterospezifisches Handhaben [*Tickling*] induzierte Rufe durch die vorherige Konfrontation mit Stress durch Bewegungseinschränkung [*Restrain*] deutlich verringert wurden (Popik et al. 2012; Popik et al. 2014), ebenso wie durch Hunger, helles Licht oder Raubtiergeruch (Panksepp und Burgdorf 2010). Auch die Antizipation speziesspezifischen Spielverhaltens ist anfällig für aversive Bedingungen, da helles Licht die Emission sozial motivierter 50-kHz USV abschwächt (Knutson et al. 1998). Allerdings hat der dämpfende Effekt aversiver Kontexte seine Grenzen. War die soziale Motivation der Tiere durch vorhergehende soziale Isolation erhöht, wirkte sich die elektrische Stimulation nicht merkbar auf die separationsinduzierten 50-kHz USV aus (Rojas-Carvajal und Brenes 2020). Die kumulierte 50-kHz USV-Produktion nach 24h sozialer Isolation war im Home Cage Setting zwischen zuvor gestressten und nicht gestressten Individuen vergleichbar hoch (Rojas-Carvajal und Brenes 2020). Bei genauerer Betrachtung der initialen 50-kHz USV-Produktion in den ersten Minuten der Testung zeigt sich jedoch, dass gestresste Individuen auch hier weniger 50-kHz USV emittierten als ihre nicht gestressten Artgenossen. Hierzu finden sich jedoch keine statistischen Angaben der Autor:innen selbst. Wenngleich konzeptuell durchaus abweichend definiert, führt Stress demnach zu einer vergleichbaren Abnahme der 50-kHz USV und bekräftigt somit die Theorie der aversiv bedingten Verringerung von 50-kHz USV.

Die Effekte vermeintlich anxiolytischer Substanzen auf die 50-kHz USV-Produktion unterscheiden sich je nach Untersuchungskontext stark. In Untersuchungen der isolationsinduzierten 50-kHz USV-Produktion während speziesspezifischer Interaktion riefen Anxiolytika teilweise eine vermehrte Rufproduktion hervor (Hamed et al. 2009). Während Diazepam bei akuter Gabe die 50-kHz USV-Produktion verstärkte, zeigte der 5-HT_{1A}-Antagonist Buspiron keine Effekte (Hamed et al. 2009). Beiden Pharmaka gemein ist die anxiolytische Wirkung in Mensch und Tier (Altamura et al. 2013). Während Diazepam unvermittelt agonistisch über die Stimulation der GABAa-Rezeptoren wirkt (Möhler 2006), benötigt Buspiron, ähnlich anderer serotonerg wirkender Antidepressiva und Anxiolytika, adaptive Veränderungen der 5-HT_{1A}-Rezeptoren, um seine anxiolytische Wirkung zu entfalten (Blier und Ward 2003; Goa und Ward 1986). Diese unterschiedlich schnellen Wirkmechanismen könnten die unterschiedlichen Effekte akuter Gabe auf isolationsinduzierte 50-kHz USV bedingen. Unabhängig der Wirkmechanismen bleibt festzuhalten, dass sich der Einfluss GABAerger Transmission anders auf die Rufproduktion auswirkt als serotonerge Manipulationen.

Da USV meist in einem funktionalen Zusammenhang gesehen werden und nicht „um ihrer selbst willen“ (Brudzynski 2021) produziert werden, stellt sich die Frage, welche Funktion der Rückgang von 50-kHz USV in aversiven Kontexten haben kann. Gemeinhin sind 50-kHz USV mit Belohnung (Burgdorf et al. 2007) und der Wiederherstellung von Sozialkontakt (Wöhr et al. 2008; Wöhr und Schwarting 2013) assoziiert. Untersuchungen zeigen, dass vor allem unmittelbar nach der Trennung der Tiere vor allem flache 50-kHz USV dominieren (Rao und Sadananda 2015; Wöhr et al. 2008). Interessanterweise sind Befunde zu 50-kHz USV-Emission unter Entzugserscheinungen ebenso von sozialen Komponenten konfundiert (Vivian und Miczek 1991). Es ist also durchaus denkbar, dass auch vermeintlich neutrale Umgebungen ohne explizite soziale Hinweisreize Komponenten der sozialen Motivation evozieren, die durch die Beschaffenheit der Umgebung weiterhin begünstigt wird. Erwiesenermaßen vokalisierten Ratten vermehrt, wenn sie in einer Umgebung mit Einstreu getestet wurden (Brenes et al. 2016; Natusch und Schwarting 2010). Dieser 50-kHz potenzierende Effekt ist resistent gegen Einflüsse von Stress, da Tiere in einem Käfig mit Einstreu im Vergleich zu einem uneingestreuten Offenfeld keine stressbedingten Verringerungen der Rufrate zeigten (Rojas-Carvajal und Brenes 2020). Ob dieser Effekt auf der Vertrautheit [*Familiarity*] mit der Umgebung und dem damit assoziierten Sozialkontakt zurückgeht, oder Tiere in Umgebungen mit Einstreu eher ein von ihnen präferiertes Aktivitätsmuster [*Prefered Activity Pattern*, Fanselow und Lester 1988] zeigen können, bleibt zu klären. Der Rückgang der 50-kHz USV in vergleichbar aversiven Kontexten kann demnach mit

einem weniger angenehmen emotionalen Zustand oder/und dem daraus resultierenden, geringeren sozialen Interesse koinzidieren.

Zudem gibt es Hinweise, dass die dopaminerge Innervation für Angstverhalten durchaus eine Rolle spielt (für Übersicht: de la Mora et al. 2010), die somit die Schnittmenge zwischen anxiogenen Effekten und der verringerten 50-kHz USV-Produktion der Studie I darstellen könnte. De la Mora (2010) fasst hierbei zusammen, dass topographisch nicht überlappende Dopamin-D1- und D2-Rezeptor-Bindungsstellen das Individuum auf die Bewältigung realer oder potenzieller Bedrohungen aus seiner Umwelt vorbereiten können. Hierbei sei der D1-Rezeptor in der basolateralen Amygdala (BLA) entscheidend für die Gefahrenerkennung, da er den Abruf affektiver Eigenschaften unkonditionierter Umweltreize erleichtere und somit sowohl konditionierte als auch unkonditionierte Assoziationen begünstige. Dopamin-D2-Rezeptoren könnten stattdessen an der Modulation reflexartiger Verhaltensweisen im Hirnstamm und an der Entwicklung adaptiver Reaktionen zur Bewältigung aversiver Umweltsituationen beteiligt sein.

4.1.2 22-kHz USV in bedrohlichen Kontexten

4.1.2.1 Bedrohung vs. Konditionierung

Wie zu erwarten war, ist die Präsenz aversiver Stimuli wie elektrische Stimulation, konditionierte Furchtreize oder Raubtiergeruch mit 22-kHz USV-Emission verbunden (Brudzynski 2021, 2007; Litvin et al. 2007). Die Prävalenz furchtinduzierter 22-kHz USV wird maßgeblich von der Nähe akuter Bedrohung beeinflusst und variiert mit der Aversivität der Situation. Hierbei ist die akute Bedrohung durch elektrische Stimulation mit der höchsten 22-kHz USV-Produktion assoziiert (Studie III und IV), während konditionierte Reize (Studie III und IV) oder olfaktorische Hinweisreize (Studie II) weniger 22-kHz USV evozieren. Der Großteil der zu beobachtenden Rufe wurde während der direkten Konfrontation mit der elektrischen Stimulation (Akquisition) emittiert, wohingegen die alleinige Konfrontation mit dem konditionierten Stimulus (Extinktion) lediglich einen Bruchteil der Rufe evoziert (Studie IV) – oder die 22-kHz Rufproduktion gänzlich zum Erliegen kommt (Studie III) – diese Effekte waren weiterhin stark beeinflusst von Genotyp und Geschlecht (siehe nachfolgende Kapitel). In früheren Studien mit Furchtkonditionierungsprotokollen identisch zu Studie III fanden sich vergleichbare Muster (Borta et al. 2006; Wöhr et al. 2005; Wöhr und Schwarting 2008a, 2008b; Yee et al. 2012a, 2012b) und auch andere Furchtkonditionierungsprotokolle zeigen, dass Ratten an Testtagen ohne

elektrische Stimulation weniger 22-kHz USV emittierten (Hegoburu et al. 2011; Kassai und Gyertyán 2012; Koo et al. 2004).

Im Vergleich zu der 22-kHz USV-Prävalenz der Furchtkonditionierung ist die geruchsinduzierte 22-kHz USV-Produktion verhältnismäßig schwächer als die 22-kHz USV-Produktion während der Akquisition und stärker als zum Zeitpunkt der Extinktion (siehe Abbildung 4), folgt aber dennoch demselben Prinzip der bedrohungsassoziierten Abnahme. In Abhängigkeit seiner, um ein Vielfaches geringeren Kairomon-Konzentration, löst Fuchsurin ungleich weniger Rufverhalten aus als Löwenurin (Studie II) und deckt sich mit der Annahme, dass die zunehmende Unmittelbarkeit der Bedrohung mit einem vermehrten Abwehrverhalten (Fanselow und Lester 1988) – in Form von 22-kHz USV – einhergeht. Perspektivisch würde ein systematischer Vergleich der intraindividuellen Vokalisationsneigung über diverse 22-kHz USV induzierende Situationen helfen, die situationsbedingten Effekte auf das USV-Spektrum eingehender zu untersuchen.

4.1.3 Angst vs. Furcht – Verortung im USV Spektrum

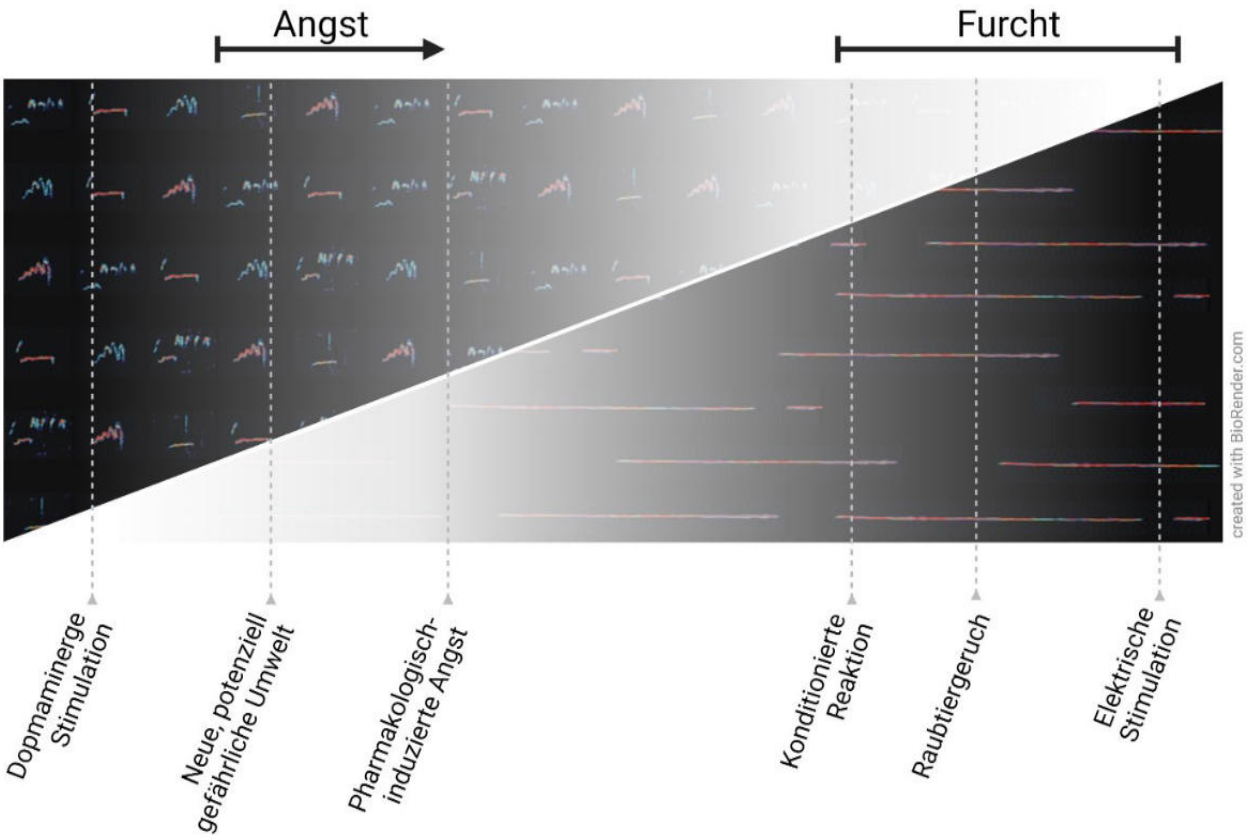
In Abhängigkeit verschiedener situationsspezifischer Faktoren lassen sich die Tiere an unterschiedlichen Stellen des USV-Spektrums verorten. Ungefährliche und neue Umgebungen gehen mit moderaten Rufraten von 50-kHz USV einher, die sich durch pharmakologische Manipulationen in die eine oder die andere Richtung beeinflussen lassen. Stimulierende und anxiolytische Substanzen führen hierbei zu einer vermehrten Rufproduktion (Burgdorf et al. 2007; Engelhardt et al. 2018; Hamed et al. 2009; Rippberger et al. 2015; Simola et al. 2012; Thompson et al. 2006; Wintink und Brudzynski 2001), während nachgewiesenermaßen anxiogene Substanzen eine abschwächende Wirkung auf die Emission von 50-kHz USV haben (Studie I).

Am gegenüberliegenden Ende des Spektrums befinden sich mit einer vermeintlich akuten Bedrohung assoziierte Situationen, wie dem Geruch von Fressfeinden (Studie II), elektrische Stimulation oder damit assoziierte Stimuli (Studie III und IV). Wie bereits erwähnt, riefen diese Testsituationen zuverlässig 22-kHz USV hervor, wobei die schockinduzierten 22-kHz USV den Endpunkt der maximal möglichen 22-kHz USV-Emission bildeten, die sich wiederum mit Abnahme der Bedrohung verringerte.

Um die durch situationsspezifische Faktoren bedingten Verhaltenseffekte der vorliegenden Studien I bis IV systematisieren zu können, soll die vorher aufgeführte Dichotomie von Angst und

Furcht in Anlehnung an das *Predatory Imminence Continuum* (Fanselow und Lester 1988) mit dem Spektrum der USV in Verbindung gebracht werden. Hierbei werden ungewisse, distale und potenzielle Gefahrensituationen [*Pre-encounter Phase]* mit Angst und einer dementsprechenden Verringerung bevorzugter Verhaltensweise assoziiert. Eine Verringerung der 50-kHz USV im Zusammenhang mit zunehmend aversiven Kontexten wäre dementsprechend ein Hinweis auf einen affektiven Zustand ähnlich dem der Angst. Es ist davon auszugehen, dass die Emission von 50-kHz USV eine von den Tieren bevorzugte Verhaltensweise, ähnlich der Nahrungsaufnahme oder dem Explorationsverhalten, darstellt und durch die zunehmende Ungewissheit der potenziellen Gefahrensituation unterdrückt wird (siehe Abbildung 4). Auf der anderen Seite wird die Detektion einer realen, externen Bedrohung [*Post-encounter Phase]* wie beispielsweise dem Geruch von Fressfeinden oder dem Vorhandensein von elektrischer Stimulation mit speziesspezifischem Abwehrverhalten und damit einhergehender Furcht assoziiert. Die Emission von 22-kHz USV im direkten Zusammenhang mit dem Vorhandensein von realen Gefahren könnte demnach als Indikator für einen furchtsamen Zustand gelten (siehe Abbildung 4).

Abbildung 4. Situationsspezifische Faktoren, Angst und Furcht im USV-Spektrum.



Ein weiterer elementarer Bestandteil das *Predatory Imminence Continuum* und somit entscheidend für die Verortung der USV-Emission in Anlehnung daran, ist die Beachtung des panikähnlichen Fluchtverhaltens (*circa-strike behavior,* Fanselow und Lester 1988), das durch unmittelbaren Kontakt mit dem realen Fressfeind oder mit der elektrischen Stimulation evoziert wird. Eine wesentliche Unterscheidung im Sinne des *Predatory Imminence Continuum*, deren Vernachlässigung zu anderen Rückschlüssen auf den emotionalen Gehalt der 22-kHz USV-Emission führt. So postulieren Brudzynski (2021) und Jelen et al. (2003) beispielsweise, dass 22-kHz USV-Emissionen mit einem Zustand der Angst und ausdrücklich nicht mit dem der Furcht in Verbindung gebracht werden können. Brudzynski (2021) argumentiert im Sinne des *Predatory Imminence Continuum*, dass der Zustand der Angst mit 22-kHz USV assoziiert ist, während hörbare [*audible*] Rufe und Stille die Begleiter einer Furchtreaktion sind und als Warnung gegen den Fressfeind emittiert würden (nach Litvin et al. 2007). Jelen et al. (2003) beobachtete, dass Hinweisreize für Gefahr zu einer Beendigung der 22-kHz USV führen, wohingegen Sicherheitssignale diesen Effekt umkehrten. Auf Grund seiner Assoziation mit der distinkten Bedrohung der elektrischen Stimulation (unkonditionierter Stimulus, US) wurde der Gefahrenreiz [*Danger Signal*, DS], der einem unvermeidlichen Schwanzschock vorausging, als konditionierter Stimulus verstanden, der im Allgemeinen Furcht auslöst. Das Sicherheitssignal (SS), das das Ausbleiben der elektrischen Stimulation anzeigte, hingegen hemme die konditionierte Furcht und wurde von 22-kHz USV begleitet, die über die Intertrial-Intervalle hinaus stabil waren. Die Produktion von 22-kHz USV während SS und zwischen den Testdurchläufen wurde dementsprechend als angstähnliche Reaktion auf unbestimmte Gefahrensituation interpretiert, deren Ausbleiben in Anwesenheit des DS dementsprechend als Furchtreaktion. Es ist allerdings fraglich, ob das verwendete Paradigma von Jelen die mit vagen Situationen assoziierte angstähnliche Reaktionen und den mit spezifischen Bedrohungen einhergehenden furchtsamen Zustand widerspiegelt oder ob hier vielmehr ein PTBS-ähnliches Design vorliegt. Die Intensität der elektrischen Stimulation ist in diesem Paradigma bedeutend höher (3 mA) als die üblicherweise verwendeten Intensitäten anderer Furchtkonditionierungsparadigmen, die im Durchschnitt zwischen 0,4 und 0,6mA liegen (Übersicht: Bali und Jaggi 2015; Studie II und IV; Borta et al. 2006; Kassai und Gyertyán 2012; Koo et al. 2004; Kosten et al. 2005; Kosten et al. 2006; Wöhr et al. 2005; Wöhr und Schwarting 2008b; Yee et al. 2012a, 2012b). Schockintensitäten zwischen 1,5 und 2mA hingegen würden in Tiermodellen der PTBS verwendet (Bali und Jaggi 2015) und werden ab einer Intensität von 3mA bereits als traumatisierende Erfahrungen klassifiziert (Mikics et al. 2008). Neben den hohen Intensitäten der elektrischen Stimulation in der Konditionierungsphase finden sich zusätzliche, aversive Bedingungen des Studiendesigns, die in ähnlicher Form auch in Tiermodellen der PTBS

verwendet werden. Einerseits wird neben der Applikation der DS-US Paarung zu Beginn der Testung, die Konfrontation mit der elektrischen Stimulation über mehrere Wochen fortgeführt. Ähnliche wöchentliche Wiederholungen finden sich in Untersuchungen zum Tiermodell der PTBS von Pynoos et al. (1996). Andererseits sind die Tiere während der gesamten Testung durch den Aufbau der Testapparatur bewegungseingeschränkt [*restrained*]. Auch der durch Bewegungseinschränkung induzierte Stress wird in Tiermodellen zur PTBS als wirksamer Auslöser traumatisierender Erfahrungen diskutiert (Souza et al. 2017), meist in Verbindung mit elektrischer Stimulation (Schöner et al. 2017). Es ist demnach durchaus denkbar, dass die Verhaltensänderung aufgrund der Darbietung von DS und SS nicht den Übergang zwischen Frucht und Angst markiert, sondern die Tiere von panikähnlichem Fluchtverhalten (evoziert durch den DS) zu einer konditionierten Furchtreaktion vor dem gesamten, mit Aversion assoziierten Testkontext während des SS übergehen. Panikähnliche Zustände, beispielsweise aufgrund nahender Fressfeinde, werden in Ratten mit hörbaren Vokalisationen assoziiert (Litvin et al. 2007), die bei elektrischer Stimulation einer Intensität zwischen 2,5 und 5 mA einsetzen (Jourdan et al. 1995) und bereits bei niedrigeren Intensitäten in direktem Anschluss an die Stimulation zu beobachten sind (unveröffentlichte Beobachtungen aus Studie III und IV). Es ist denkbar, dass die 22-kHz USV-Emission bei höheren Schockintensitäten aufgrund panikähnlicher Zustände evoziert durch den DS einer hörbaren Vokalisation weicht. Informationen diesbezüglich werden von Jelen et al. leider nicht berichtet und bleiben somit Gegenstand der Spekulation. Zusammengenommen wären demnach angstähnliche Zustände mit einer verringerten 50-kHz USV-Emission, furchtähnliche Zustände mit 22-kHz USV und panikevozierende Situationen mit hörbaren Rufen assoziiert.

Das Verhältnis von 50- und 22-kHz USV und somit der Übergang zwischen verschiedenen affektiven Zuständen wird hierbei von den situationsspezifischen Begebenheiten beeinflusst und ist nicht zwangsläufig linear. In verschiedenen Untersuchungen, die sich beide Rufarten gleichzeitig anschauen, werden meist keine Korrelationen zwischen 50- und 22-kHz USV gefunden (Mällo et al. 2007a) oder nicht berechnet (Rojas-Carvajal und Brenes 2020; Taylor et al. 2017; Yee et al. 2012a). Tickling-induzierte 50- und 22-kHz USV stehen in keinem statistischen Zusammenhang und scheinen demnach keine einander ausschließenden Kategorien abzubilden, sondern vielmehr verschiedene (affektive) Aspekte der Tickling-Prozedur widerzuspiegeln (Mällo et al. 2007a). Befunde zur stressbedingten Reduktion von 50-kHz USV bei gleichzeitig vermehrter 22-kHz USV-Produktion nach elektrischer Stimulation zeigen zeitliche Überschneidungen (Rojas-Carvajal und Brenes 2020). Allerdings lässt sich hier keine qualifizierte Aussage über den individuellen Verlauf der Rufproduktion treffen, da sich aus den berichteten Ergebnissen von

Rojas-Caval et al. (2020) keine Darstellung der Zusammenhänge von 50- und 22-kHz ableiten lassen. Es ist also denkbar, dass sich verschiedene Konfigurationen von Rufmustern innerhalb der Testung finden ließen: 1) Tiere, die nur 50-kHz USV emittieren, 2) Tiere, die nur 22-kHz USV emittieren und 3) Tiere, die beide Ruftypen gleichzeitig oder 4) einen Übergang von 50- zu 22-kHz USV zeigen. Es gibt Hinweise, dass in zunehmend aversiven Bedingungen, wie der wiederholten Verabreichung elektrischer Stimulation, ein Übergang von 50-kHz USV hin zu 22-kHz USV zu beobachten ist, der den Übergang von einem ängstlichen in einen furchtsamen Zustand markieren soll (Taylor et al. 2017; Yee et al. 2012a). Zudem scheint es eine Dosis-Wirkungs-Beziehung zwischen der Häufigkeit und Intensität der elektrischen Stimulation und dem Übergang zwischen 50- und 22-kHz USV zu geben. Bei einmaliger Konfrontation mit einem milden elektrischen Reiz (0,4 mA) ist bereits eine Verringerung der 50-kHz USV-Emission ohne das Einsetzen von 22-kHz USV zu verzeichnen (Burgdorf et al. 2000; Reyes et al. 2021). Interessanterweise beeinflusst die pränatale Aktivierung des maternalen Immunsystems die 22-kHz USV während der Furchtkonditionierung, jedoch nicht die 50-kHz USV-Emission in diesem Zusammenhang (Yee et al. 2012a) und stützt somit die These teilweise unabhängiger Regulation von Angst und Furcht und der damit einhergehenden USV.

Bemerkenswerterweise stellt sich jedoch auch nach erfolgreicher Furchtextinktion (gemessen an der Reduktion der furchtinduzierten 22-kHz USV-Produktion und Immobilität) keine Rückkehr zum 50-kHz USV-Ausgangsniveau ein (eigene Beobachtungen der Studie III und IV). Das Ausbleiben der vor der Furchtkonditionierung gezeigten Verhaltensweisen steht im Einklang mit der Annahme, dass die Furchtextinktion keine bloße Löschung des zuvor mit Furcht assoziierten Verhaltens ist, sondern das Ergebnis inhibitorischen Lernens darstellt (Bouton 1994, 2002), das nicht zwangsläufig die Wiederherstellung des Ausgangsverhaltens bedeutet.

Neben den situativen Einflüssen unterscheiden sich Angst und Furcht zudem in ihren neuronalen Korrelaten (Perusini und Fanselow 2015; Steimer 2002), die wiederum teilweise Entsprechungen in den zugehörigen Rufarten finden. Während spezifische Stimuli wie Töne, Licht oder Berührung die Amygdala aktivieren, sprechen weniger spezifische und komplexere Umgebungen eher den BNST an (Davis 1998). Läsionen der Amygdala unterdrücken Freezing, während das Angstlevel im erhöhten Plus-Labyrinth davon unberührt bleibt (Treit et al. 1993). Vor allem die CeA wird mit der Expression furchtinduzierten Verhaltens in Verbindung gebracht (Killcross et al. 1997; Cain und LeDoux 2008) und unterdrückt bei chirurgischer Entfernung 22-kHz USV (Choi und Brown 2003). Hinzu kommt der Einfluss des von der Amygdala angesteuerten PAG, das maßgeblich für das Freezing ist (Panksepp 2005). In einem Dosis-Wirkungs-Zusammenhang, verändert sich bei zunehmender Stimulation das Freezingverhalten entsprechend dem P*redatory Imminence*

Continuum und wechselt von Wachsamkeit [*Alertness*] über Freezing zu Flucht (Vianna et al. 2001). Die pharmakologische Stimulation des PAG wirkt sich ebenso verstärkend auf die Produktion von 22-kHz USV aus (Depaulis et al. 1992). Auch das Playback dieser Rufe spricht diese Struktur an (Sadananda et al. 2008). Es scheint demnach eine bedeutende Überschneidung der gängigen neuronalen Korrelate des Furchtverhaltens und 22-kHz USV zu geben, da sowohl Amygdala, PAG mit furchtinduzierter Immobilität und USV-Produktion assoziiert werden.

Das Verhältnis von neuronalen Korrelaten der Angst und 50-kHz USV ist jedoch nicht im gleichen Maße ausgeprägt wie die von Furcht und 22-kHz USV. Gemeinhin sind 50-kHz USV im Zusammenhang mit dopaminerger Transmission untersucht worden (Übersicht: Brudzynski 2021). Es gibt jedoch bereits Hinweise auf einen Einfluss des BNST auf CRH-vermittelte 50-kHz USV (Taylor 2017). Eine detaillierte pharmakologische Untersuchung in neutralen bis leicht aversiven, anxiogenen Kontexten, und somit eine Trennung der situationsspezifischen 50-kHz USV an Hand neuronaler Korrelate steht noch aus.

4.2 Einfluss biologischer Faktoren

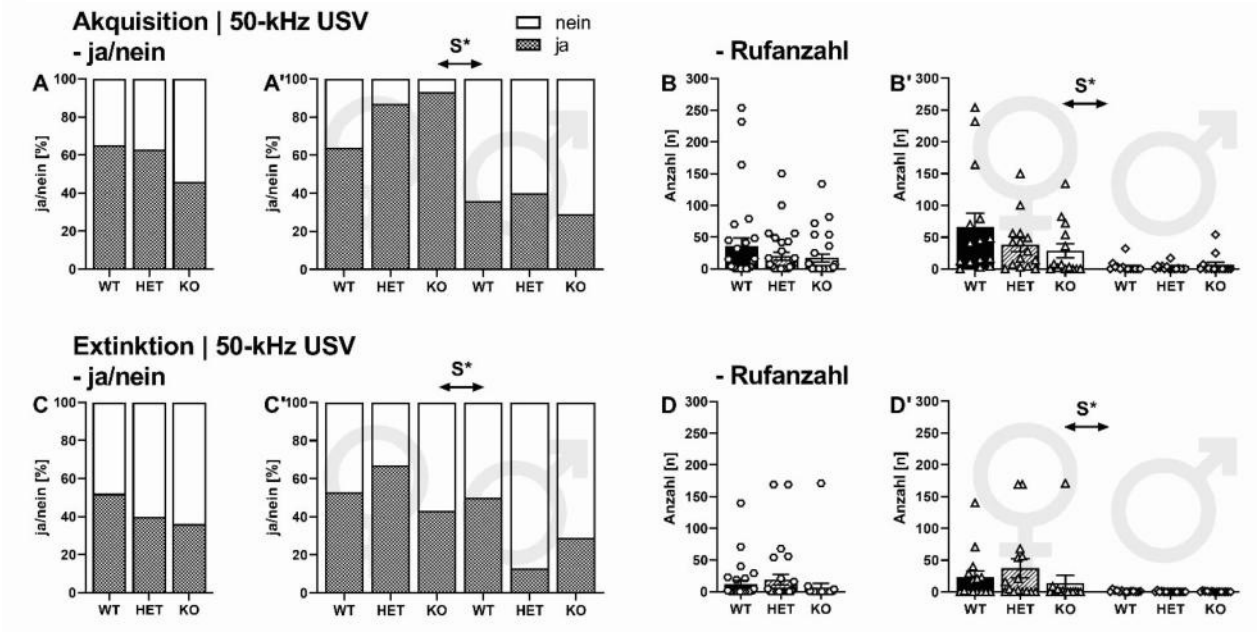
4.2.1 Geschlechtseffekte

4.2.1.1 50-kHz USV in neuen, potenziell gefährlichen Umgebungen

Um den gefundenen Geschlechtsunterschiede in nicht-sozialen, neuen Kontexten der Studie III replizieren zu können, wurden ergänzende Analysen zu Studie IV durchgeführt (siehe Abbildung 5). Vor allem in der Phase der initialen Konfrontation mit einer neuen Umgebung während der Habituationsperiode wurden in beiden Untersuchungen deutlich mehr 50-kHz USV von weiblichen Ratten produziert als von ihren männlichen Artgenossen. Da die Emission von 50-kHz USV meist in Verbindung mit Belohnung oder Sozialverhalten untersucht wird, gibt es nur äußerst wenige Berichte über ähnliche Befunde. Einzig Taylor et al. (2017) fanden übereinstimmende Geschlechtseffekte in der frühen Phase der Furchtakquisition, in der Weibchen signifikant mehr 50-kHz USV produzieren.

Dieser Geschlechtseffekt konzentriert sich vorrangig auf die Habituationsphase des Furchtkonditionierungsparadigmas, da die Rufraten beider Geschlechter im Offenfeld keine bedeutsamen Unterschiede aufwiesen (eigene Beobachtungen). Welche Bedingungen die erhöhte Rufrate der Weibchen begünstigen, bleibt abschließend zu klären, da sich Offenfeld und Furchtkonditionierung in vielen Punkten wesentlich unterscheiden: 1) Lichtverhältnis: Die

Abbildung 5 Prävalenz und Anzahl 50-kHz USV während der Furchtkonditionierung.



Anmerkung. Effekte von Geschlecht und Genotyp auf die Prävalenz (A, A'; ja – karierter Balken; nein – weißer Balken) und die Anzahl (B, B') von 50-kHz USV während der Akquisition, und auf die Prävalenz (C, C') und Anzahl (D, D') von 50-kHz USV während der Extinktion. Prävalenz und Anzahl werden gezeigt für SERT-WT (schwarzer Balken), -HET (gestreifter Balken) und -KO (weißer Balken) mit gepooltem Geschlecht (A, B, C, D) und getrennt für Geschlecht (A', B', C', D') mit Weibchen auf der linken und Männchen auf der rechten Seite der Abbildung. N = 44 weibliche Ratten (15 WT, 15 HET, 14 KO), N = 43 männliche Ratten (14 WT, 15 HET, 14 KO). Daten werden als Mittelwerte ± Standardfehler präsentiert. S* $p < 0.05$ Effekt des Geschlechts.

In zusätzlichen Analysen wurde gezeigt, dass die 50-kHz USV-Emission während der Furchtakquisition maßgeblich vom Geschlecht beeinflusst wurde, mit einer vermehrten 50-kHz USV-Produktion der Weibchen (S: $chi^2_2 = 19.749$, $p < 0.001$). Während 82% (N = 36 von N = 44) der Weibchen 50-kHz USV emittieren, tun dies nur 35% (N = 15 von N = 43). Es wurden keine Effekte des Genotyps auf die 50-kHz USV-Emission während der Akquisition gefunden (G: $chi^2_2 = 2{,}559$, $p = 0.278$). Dementsprechend unterscheidet sich die Anzahl produzierter 50-kHz USV zwischen den Geschlechtern, jedoch nicht in Abhängigkeit des Geschlechts (S: $F_{1,87} = 19.067$, $p < 0.001$; G: $F2{,}87 = 1.402$, $p = 0.252$; GxS: $F_{2,87} = 1.653$, $p = 0.198$). Während der Extinktionsphase wurde die 50-kHz USV Emission ebenfalls vom Geschlecht beeinflusst, mit einer vermehrten 50-kHz USV-Produktion der Weibchen (S: $chi^2_2 = 5{,}259$, $p = 0.022$). Während 55% (N = 24 von N = 44) der Weibchen 50-kHz USV emittieren, tun dies nur 30% (N = 13 von N = 43) der Männchen. Es wurden keine Effekte des Genotyps auf die 50-kHz USV Emission gefunden (G: $chi^2_2 = 1{,}614$, $p = 0.446$). Während der Extinktion unterscheidet sich die Anzahl produzierter 50-kHz USV zwischen den Geschlechtern, jedoch nicht in Abhängigkeit des Genotyps (S: $F_{1,87} = 10.771$, $p = 0.002$; G: $F_{2,87} = 0.897$, $p = 0.412$; GxS: $F_{2,87} = 0.926$, $p = 0.400$).

Testung im Offenfeld findet unter Rotlicht statt, die Furchtkonditionierung unter Weißlicht; 2) Bodenbeschaffenheit: Im Offenfeld befindet sich frisches Einstreu, der Boden der Furchtkonditionierungsapparatur besteht aus Edelstahlstangen; 3) im Offenfeld werden mehrere Tiere gleichzeitig – wenn auch akustisch und optisch voneinander getrennt – getestet, während der Furchtkonditionierung befindet sich lediglich das Testtier im Testraum. Zusammengenommen könnte sich demnach die Beschaffenheit der Tests in ihrer Neuheit und Ungewissheit für die Tiere unterscheiden. Neben den bereits thematisierten Effekten von Einstreu auf die 50-kHz USV-Produktion (Brenes et al. 2016; Natusch und Schwarting 2010) gibt es bereits

Hinweise darauf, dass die Präferenz für Neuheit selbst einen Einfluss auf die 50-kHz USV-Produktion hat. Ratten, die selektiv für ihre Präferenz für neue Umgebung gezüchtet werden, zeigen eine verstärkte Neigung zur Tickling-induzierten 50-kHz USV-Emission (Mällo et al. 2007b). Ob sich Männchen und Weibchen in ihrer prinzipiellen Reaktion auf neue Umgebungen und dementsprechend in ihrer Grundrate der neuheitsinduzierten 50-kHz USV unterscheiden, bleibt zu klären.

Gemischte Effekte des Geschlechts auf 50-kHz USV zeigten sich auch in Kontexten mit sozialen Komponenten. Einerseits reagierten Weibchen stärker auf anästhesierte gleichgeschlechtliche Partner (Blanchard et al. 1993) und zeigten erhöhte Tickling-induzierte 50-kHz USV (Kosten et al. 2005). Auch die Annäherungs- und Vermeidungsreaktion auf wiedergegebene 50- bzw. 22-kHz USV von Artgenossen fiel bei Weibchen höher aus (Willadsen et al. 2014). Andererseits ist jedoch die 50-kHz USV-Produktion von Weibchen während des interspezies-spezifischen Spiels geringer ausgeprägt (Himmler et al. 2014; Lukas und Wöhr 2015; Kisko et al. 2021; Panksepp und Burgdorf 2003; Potasiewicz et al. 2019), sodass eine abschließende Beurteilung der geschlechtsabhängigen Unterschiede in der 50-kHz USV-Emission weiterhin aussteht.

4.2.1.2 22-kHz USV in bedrohlichen Situationen

Sowohl Studie III und IV zeigten deutliche Unterschiede in der furchtinduzierten 22-kHz USV-Produktion zwischen den Geschlechtern. Verglichen mit ihren männlichen Artgenossen ist der Anteil an vokalisierenden Weibchen erheblich reduziert. Die ungleich niedrigere Prävalenz weiblicher 22-kHz USV-Produktion während der Akquisition (Studie III und IV) wird gefolgt von der nahezu völligen Abwesenheit jeglicher 22-kHz USV-Emission in den Phasen der Extinktion (Studie III und IV), und Recovery (Studie IV). Ein möglicher Zusammenhang mit der geschlechtsabhängigen Schmerzsensitivität ist denkbar, allerdings sind die entsprechenden Befunde beider Studien hierzu nicht eindeutig. Während Studie III eine höhere Sensitivität gegenüber thermaler Stimulation bei den weiblichen Tieren feststellt, ist die Befundlage in Studie IV gegenteilig. Interessanterweise gibt es ähnliche Befunde zu höherer Sensitivität gegenüber elektrischer Stimulation von Weibchen, in denen niedrigere Stimulationslevel mit Schmerz assoziierte, hörbare Rufe in Weibchen hervorrufen, während ihre männlichen Artgenossen dieses Verhalten erst bei höheren Stimulationsleveln zeigten (Kosten et al. 2005). Allerdings zeigten sich in der Untersuchung von Kosten (2005) ähnliche, geschlechtsabhängige Befunde zum 22-kHz USV-Rufverhalten. Auch hier emittierten Weibchen in der direkten Konfrontation mit elektrischer

Stimulation weniger 22-kHz USV – bei vergleichbaren Immobilitätsleveln (Näheres zum Verhältnis von USV und Lokomotion in Kapitel 4.2.3). Es bleibt also zu klären, ob sich Weibchen bei gleicher Stärke der elektrischen Stimulation bereits in dem panik-ähnlichen Bereich des *Predatory Imminence Continuum* befinden, der von fluchtähnlichen Verhaltensweisen wie Sprüngen und hörbaren Rufen geprägt ist und zu einem Abbruch der 22-kHz USV-Emission führt (Jelen et al. 2003). Die Befunde zur Schmerzsensitivität aus Studie III sprechen dafür, die Befunde aus Studie IV dagegen. Die abschließende Klärung des Verhältnisses von 22-kHz USV, Sensitivität gegenüber elektrischer Stimulation und der Wahrnehmung von Schmerzen und hörbaren Rufen übersteigt jedoch den Umfang dieser Arbeit und bedarf weiterer Erkenntnisse in weiblichen Ratten.

Die Befundlage anderer Untersuchungen zu geschlechtsabhängigen Unterschieden in der 22-kHz USV-Produktion ist gemischt. Unter semi-naturalistischen Bedingungen produzierten Weibchen innerhalb des Sozialgefüges deutlich mehr 22-kHz USV, wenn sie einer Katze ausgesetzt waren (Blanchard et al. 1991a; Blanchard et al. 1992; Shepherd et al. 1992). Spätere Untersuchungen unter Laborbedingungen wiesen jedoch ähnliche geschlechtsabhängige Ergebnisse wie die vorgelegten Studien auf. Während der Konfrontation mit einem Luftstoß (Inagaki und Mori 2014; Inagaki und Sato 2016) oder elektrischer Stimulation (Vry et al. 1993b; Graham et al. 2009; Kosten et al. 2005; Kosten et al. 2006) emittierten die Weibchen systematisch weniger 22-kHz USV als ihre männlichen Artgenossen. Welche Bedingungen des semi-naturalistischen Settings den entscheidenden Unterschied zu den artifiziellen Bedrohungsszenarien darstellen, bleibt zu klären. Zum einen könnte die An- bzw. Abwesenheit von Artgenossen ein Faktor sein, der unterschiedlich starke Gewichtung für Männchen und Weibchen hat, wobei das Vorhandensein eines *Audience Effect* stets kritisch gesehen wird (Wöhr und Schwarting 2008b). Darüber hinaus ist noch wenig über Geschlechtsunterschiede in den akustischen Merkmalen von 22-kHz USV bekannt, und es liegen nur wenige Daten über 22-kHz USV vor, die unter standardisierten Bedingungen als Reaktion auf Luftstöße oder elektrische Fußschocks aufgezeichnet wurden. Derzeit ist unklar, ob solche Geschlechtsunterschiede lediglich anatomische Unterschiede zwischen Männchen und Weibchen widerspiegeln, wie z. B. Körpergewicht, Lungenkapazität und Länge des Vokaltrakts (Hegoburu et al. 2011; Inagaki et al. 2012; Riede et al. 2020), oder ob sie kommunikative Funktionen erfüllen, z. B. indem sie zusätzliche Merkmale liefern, anhand derer zuhörende Artgenossen das Geschlecht der vokalisierenden Ratte bestimmen können, wie von Blanchard et al. (1992) vorgeschlagen. Der Anteil der vokalisierenden Männchen bei Konfrontation mit Raubtiergeruch (Studie II) war bedeutend geringer als der Anteil vokalisierender Männchen während der Furchtakquisition (Studie III und IV). Ob dieser Effekt auf die vermeintlich weniger

imminente Bedrohung, die das Vorhandensein Raubtiergeruchs im Vergleich zu der Applikation elektrischer Stimulation evoziert, zurückzuführen ist, oder ob männliche Ratten tendenziell weniger stark auf die Bedrohung durch Fressfeinde reagieren, bleibt in einem abschließenden Vergleich beider Geschlechter zu klären. Einerseits könnte die geruchsinduzierte 22-kHz USV-Produktion unter Laborbedingungen von Weibchen den unter naturalistischen Gegebenheiten gefundenen Effekten in Konfrontation mit einem Fressfeind (Blanchard et al. 1991a; Blanchard et al. 1992) ähneln, und Weibchen somit mehr 22-kHz USV in der Gegenüberstellung mit Raubtiergeruch vokalisieren. Möglicherweise könnten sich jedoch die artifiziellen Bedrohungsszenarien in unterschiedlicher Weise auf männliche und weibliche Tiere auswirken, sodass Weibchen weniger 22-kHz USV in der Gegenüberstellung mit Raubtiergeruch vokalisieren.

Geschlechtsunterschiede bei der durch die elektrische Stimulation hervorgerufenen 22-kHz USV können ebenso stark vom Stamm beeinflusst sein (Übersicht: Lenell 2021). Es wurde berichtet, dass männliche Sprague-Dawley-Ratten mehr 22-kHz USV produzieren als ihre weiblichen Artgenossen, während für Long-Evans-Ratten das Gegenteil gezeigt wurde (Graham et al. 2009). Interessanterweise wurden in den Studien, die in natürlicher Umgebung durchgeführt wurden, Long-Evans-Ratten verwendet, während bei Sprague-Dawley-Ratten (Kosten et al. 2006) Geschlechtsunterschiede bei der durch Fußschock ausgelösten 22-kHz USV und bei der durch Luftstoß ausgelösten 22-kHz USV bei Auszucht-Wistar- und Inzucht-F344-Ratten (Inagaki und Sato 2016) festgestellt wurden. In einem detaillierten Stammvergleich zwischen Long-Evans, Sprague-Dawley- und Wistar-Ratten über verschiedene USV Paradigmen hinweg zeigte sich ein ähnlicher Effekt des Geschlechts in der Furchtkonditionierung (Schwarting 2018a, 2018b). Bemerkenswerterweise zeigten Wistar-Weibchen hier die höchsten Indizes für angstähnliches Verhalten während der frühkindlichen Isolation vom Muttertier, wohingegen ihre furchtinduzierten 22-kHz USV geringer ausfielen als die der anderen beiden Stämme. In umgekehrter Weise werden niedrige isolationsinduzierte Rufe der Jungtiere mit hohen Raten an furchtinduzierten 22-kHz USV im Erwachsenenalter assoziiert (Wöhr und Schwarting 2008a). Dies könnte ein weiterer Hinweis darauf sein, dass Angst und Furcht teilweise unabhängig voneinander reguliert und durch unterschiedliche Mechanismen beeinflusst werden.

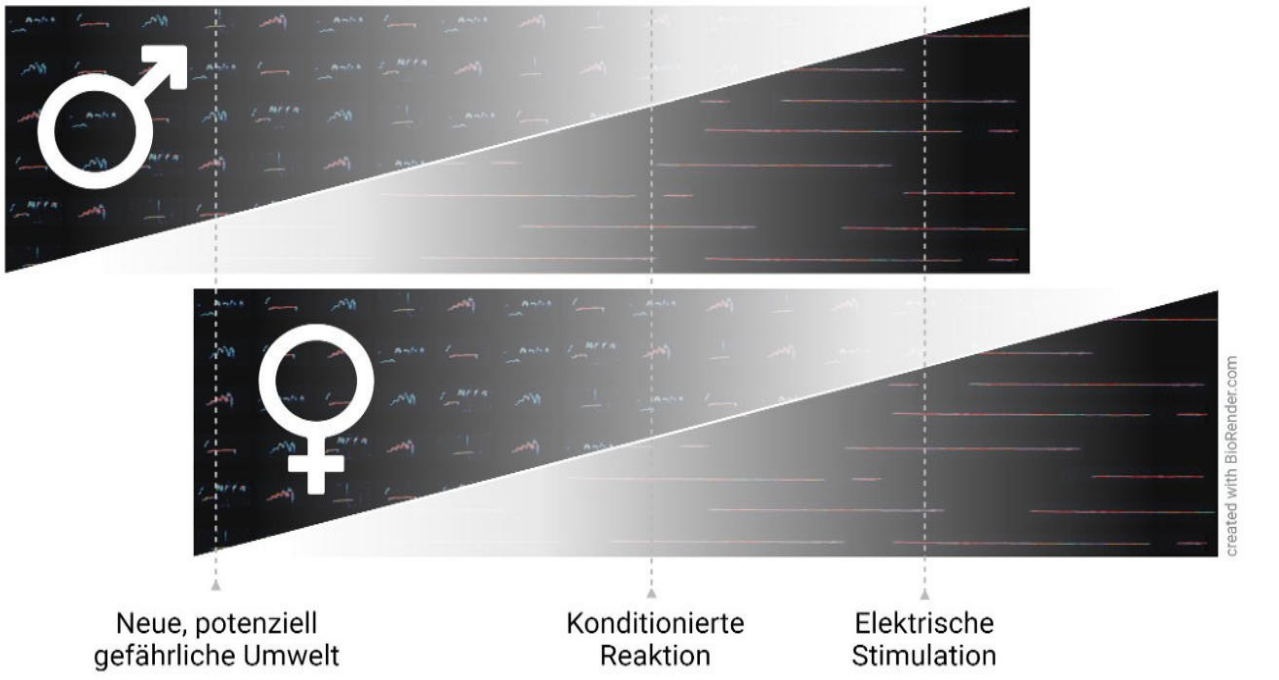
4.2.1.3 Geschlechtseffekt auf das USV-Spektrum

Die erhöhte Rufproduktion von 50-kHz USV in neutralen bis leicht aversiven Kontexten, die sich bei weiblichen Ratten beobachten lässt, steht im scheinbaren Gegensatz zu der verringerten 22-

kHz USV-Produktion als Antwort auf elektrische Stimulation. Bei Einordnung in das USV-Spektrum wird jedoch klar, dass sich die Verortung der USV evozierenden Situationen in Abhängigkeit des Geschlechts verschiebt. Weibliche Ratten behalten länger die 50-kHz USV-Produktion in neuen bis hin zu leicht aversiven Kontexten bei und gehen später in Anbetracht der realen Bedrohung durch elektrische Stimulation zur Produktion von 22-kHz USV über (siehe Abbildung 6). Übereinstimmend mit den Befunden der USV sprechen auch andere Verhaltensindikatoren von Angst und Furcht in den vorliegenden Untersuchungen für eine höhere Resilienz der Weibchen. Einerseits zeigen weibliche Ratten im erhöhten Plus-Labyrinth weniger angstähnliche Verhaltensweise wie die vermehrte Exploration der offenen Arme (Studie IV). Andererseits zeigte sich während des verlängerten Furchtkonditionierungsparadigmas in Studie IV trotz einer vergleichbaren Furchtakquisition schnelleres Extinktionslernen, das sich in weniger furchtinduziertem Immobilitätsverhalten widerspiegelt.

Weitere Untersuchungen, die das Rufverhalten beider Geschlechter über verschiedene Situationen – zum Zeitpunkt des Übergangs zwischen leicht aversiven und furchtsamen Umgebungen – sind nötig, um diese Charakterisierung zu festigen und eventuell Hinweise auf eine erhöhte Resilienz weiblicher Ratten zu liefern. Taylor (2017) berichtet bereits ähnliche Beobachtungen in einem zunehmend aversiver werdenden Kontext der Furchtakquisition, wenn auch mit einem divergierenden Fazit. Auch hier vokalisierten die Weibchen mehr 50- und weniger 22-kHz USV.

Abbildung 6. Geschlechtsabhängige Verschiebung des USV-Spektrums.



Je mehr Faktoren im Zusammenhang mit USV und geschlechtsspezifischen Unterschieden berücksichtigt werden, umso deutlicher wird der Bedarf an weiterer Forschung. Beispielsweise gibt es Hinweise auf geschlechtsspezifische Effekte stressbedingter Veränderung des Rufverhaltens von 50- und 22-kHz USV in Männchen und Weibchen, die viel oder wenig responsiv für Tickling waren (Mällo et al. 2009). In dieser Untersuchung wurden die Ratten vor Applikation eines chronischen Stresses ihrem Tickling-induziertem Rufverhalten entsprechend der hohen oder der niedrig vokalisierenden Gruppe (HC, *high calling*; LC, *low calling*) zugeordnet. Während chronischer Stress die Unterschiede in der 50-kHz USV-Emission in Männchen nivellierte, schien chronischer Stress keine merklichen Auswirkungen auf die 50-kHz USV der Weibchen zu haben. In gestressten Weibchen fanden sich weiterhin merkliche Unterschiede zwischen HC und LC nach Applikation des chronischen Stress-Regimens. Auch hier ist also festzustellen, dass Weibchen in vergleichbaren Situationen mehr 50-kHz USV produzieren. Verkompliziert wird der Zusammenhang von Geschlecht, Stress und USV-Neigung jedoch bei Betrachtung der stressinduzierten 22-kHz USV. Während männliche LC-Ratten hier durchaus der vorgeschlagenen Systematik des USV-Spektrums folgen und sich die geringe 50-kHz USV-Rate der LC-Männchen unter Einfluss von Stress in einer höheren 22-kHz USV-Produktion niederschlägt, ist das Bild der Weibchen weniger deutlich. Nach der Applikation chronischen Stresses verringert sich wider Erwarten die 22-kHz USV-Produktion bei allen Gruppen, mit Ausnahme der gestressten HC-Tiere. Aus den Informationen, die Mällo et al. (2009) zur Verfügung stellen, ist nicht ersichtlich, unter welchen Bedingungen die prä- und post-Stress-USV beobachtet wurden und welche zusätzlichen Faktoren, wie soziale Hinweisreize, Einstreu oder potenziell aversive Stimuli hinzukamen. Die potenziellen Einflussfaktoren der geschlechtsabhängigen Effekte auf das USV Spektrum scheinen vielfältig; eine vielversprechende Perspektive für weitergehende Forschungsfragen.

4.2.2 Genotypeffekte

4.2.2.1 50-kHz USV in neuen, potenziell gefährlichen Umgebungen

Das Fehlen des SERT wirkt sich teilweise auf die 50-kHz USV-Produktion aus. Während der Akquisitions- und Extinktionsphase in Studie III produzieren SERT-KO-Tiere mehr 50-kHz USV als ihre heterozygoten Artgenossen und Wildtyptiere. Allerdings findet sich dieser Effekt nicht im Paradigma der differentiellen Furchtkonditionierung der Studie IV (siehe Abbildung 5). Einer der wesentlichen Unterschiede zwischen Studie III und IV bestand in der zeitlichen Abfolge einzelner Komponenten des jeweiligen Furchtkonditionierungsparadigmas. In Studie III wurden die Tiere

24 Stunden vor der Furchtakquisition und -Extinktion an die Testapparatur gewöhnt (Ablauf siehe Borta et al. 2006; Willadsen et al. 2021a) und wurden in den entscheidenden Phasen der Furchtkonditionierung nach einer 3-minütigen Habituationsphase mit Tönen und – im Falle der Akquisition – mit elektrischer Stimulation konfrontiert. In Studie IV gab es keine Habituationsphase 24 Stunden vor der Akquisition, die Habituationsphase innerhalb der Session aller Phasen wurde jedoch auf 5 Minuten verlängert. Die unterschiedlich stark ausgeprägte Neuheit der Akquisitionsumgebung hat möglicherweise einen Einfluss auf die gemessenen 50-kHz USV, da – wie im Kapitel 4.2.1 (Geschlechtseffekte auf 50-kHz USV in neuen Umgebungen) erwähnt – die Präferenz für Neuheit und die Emission von 50-kHz USV in einem Zusammenhang stehen (Mällo et al. 2007b).

Der Einfluss des SERT-Mangels bedarf offensichtlich weiterer Untersuchungen, um robuste, interpretierbare Ergebnisse zu liefern. Obgleich 50-kHz USV-Emission meist mit dopaminerger Signaltransmission in Verbindung gebracht wird (Barker et al. 2010; Barker 2018; Brudzynski 2007, 2021; Wintink und Brudzynski 2001), gibt es Hinweise, dass Bestandteile des serotonergen Systems ebenfalls Einfluss nehmen (Überblick: Wöhr und van Gaalen 2018). Der 5-HT_{1A}-rezeptorspezifische Agonist 8-OHDPAT beispielsweise führt zu einer dosisabhängigen Steigerung der 50-kHz USV (Sadananda et al. 2012). Diese Steigerung lässt sich interessanterweise unter Gabe von MDMA, das über die Stimulation des 5-HT_{1A}-Autorezeptors (Giannaccini et al. 2007) zu einer drastischen Abnahme des zur Verfügung stehenden 5-HT führt (Slikker et al. 1989; Lowry und Hale 2010), nicht wiederfinden. Die MDMA-induzierte Stimulation des Verhaltens beschränkt sich jedoch nur auf die Hyperlokomotion (Paulus und Geyer 1992), Ultraschallvokalisationen bleiben im Gegensatz dazu unberührt (Sadananda et al. 2012). Auch die Vorbehandlung mit dem 5-HT_{1A}-rezeptorspezifischen Antagonist NAD-299 zeigt hingegen keinerlei Einfluss auf das amphetamininduzierte Rufverhalten von Tieren (Wright et al. 2012). Ähnlich den Befunden zum 5-HT_{1A}-Rezeptor, finden sich auch Hinweise auf den Einfluss des 5-HT_{2C}-Rezeptors auf 50-kHz USV. Studie I hat gezeigt, dass der 5-HT_{2C}-spezifische Agonist mCPP zu einer Abnahme von spontanen 50-kHz USV im Offenfeld führt. Während der 5-HT_{2C}-spezifische Agonist CP 809,101 das Amphetamin-induzierte Rufverhalten abschwächt und vor allem den Trill-Subtyp der frequenzmodulierten Rufe minimiert, wird es von dem entsprechenden 5-HT_{2C}-Antagonisten SB 242084 verstärkt (Wöhr et al. 2014). Auch bei alleiniger Verabreichung von SB 242084 ist eine Stimulation der 50-kHz USV zu beobachten (Wöhr et al. 2014). Verschiedene Rezeptoren des serotonergen System scheinen demnach an der Regulation von 50-kHz USV beteiligt zu sein und spielen durch ihre SERT-Mangel bedingte Veränderungen eine Rolle für die genotypabhängigen Effekte in der USV-Produktion.

4.2.2.2 22-kHz USV in bedrohlichen Situationen

Ähnlich den geschlechtsabhängigen Effekten, spielt auch der SERT-Mangel eine entscheidende Rolle in der 22-kHz USV-Produktion. Sowohl die Prävalenz, als auch die Rufdauer der SERT-KO-Ratten waren über alle Phasen des Experiments signifikant reduziert (Studie III und IV). Erwiesenermaßen führt das Fehlen des SERT bei Ratten zu einem deutlichen Anstieg des basalen extrazellulären 5-HT-Spiegel (Homberg et al. 2007a). Die Verringerung von 22-kHz USV auf Grund eines wesentlichen Anstiegs des 5-HT-Spiegels wurde von Sanchez (1993, 2003b) zusammengefasst, wobei eine Vielzahl von SSRIs der Emission von 22-kHz USV entgegenwirken. Hinzu kommen Veränderungen in der 5-HT-Rezeptorexpression oder -Empfindlichkeit. Bei SERT-KO-Ratten ist der 5-HT_{1A}-Rezeptor desensibilisiert (Snoeren et al. 2010; Olivier et al. 2008; Homberg et al. 2008) und die Funktion des 5-HT_3-Rezeptors ist verändert (El-Ayache und Galligan 2019). SERT-KO-Mäuse weisen zudem eine veränderte Bindung und Funktion von 5-HT_{1A}- und 5-HT_{1B}-Autorezeptoren auf (Alexandre et al. 2006; Fabre et al. 2000), ebenso wie eine veränderte 5-$HT_{2A/2C}$-Rezeptordichte (Qu et al. 2005). Vollständige oder partielle 5-HT_{1A}-Rezeptor-Agonisten blockieren nachweislich 22-kHz USV-Emission (Übersicht: Sánchez 2003b; Wöhr und van Gaalen 2018). Die Untersuchung verschiedener 5-HT_{1A}-Rezeptor-Agonisten, wie beispielsweise 8-OH-DPAT, Buspiron, Gepiron und Ipsapiron, zeigten, dass unter normalen Bedingungen die 22-kHz USV-Emission durch den 5-HT_{1A}-Rezeptor gehemmt wird (Vry et al. 1993b; Vry et al. 2004; Kassai und Gyertyán 2012). Ist jedoch der 5-HT_{1A}-Rezeptor wie bei den SERT-KO-Ratten desensibilisiert, wird die Inhibition geschwächt und eine vermehrte 22-kHz USV-Produktion wäre zu erwarten. Das paradoxe Zusammenspiel aus 5-HT_{1A}-Desensibilisierung und genotypabhängiger, verringerter 22-kHz USV-Emission bedarf demnach weiterer Klärung.

Weitere genotypabhängige neurobiologische Unterschiede tragen möglicherweise zu diesen Effekten der veränderten 22-kHz USV-Emission bei. Eine Schlüsselrolle beim Erwerb von Angst und Furcht nimmt hierbei die Amygdala ein (Huang et al. 2013), die durch ein Zusammenspiel mehrerer Kerne die Produktion von 22-kHz USV steuert (Furtak und Brown 2018). Einerseits vermitteln sowohl der mediale Kern (McCue et al. 2014) als auch der basolaterale Kern der Amygdala (Hamdani und White 2011) die 22-kHz USV-Produktion während des konditionierten Vermeidungsverhaltens. Andererseits hat der CeA die bedeutendsten Auswirkungen auf die 22-kHz USV-Produktion (Furtak und Brown 2018). Wie bereits erwähnt, blockiert die Entfernung (Choi und Brown 2003) oder neurotoxische Läsion (Koo et al. 2004) der CeA die 22-kHz USV-Emission vollständig. Interessanterweise weisen SERT-KO-Ratten eine abweichende neuronale

Aktivität der CeA auf (Shan et al. 2018), was zu Veränderungen in der 22-kHz USV-Produktion beitragen könnte.

Neben den direkten Effekten des serotonergen Systems auf die Produktion von 22-kHz USV ist 5-HT stark an der Regulierung des Sozialverhaltens beteiligt, da ein SERT-Mangel zu sozialen Defiziten führt (Kiser et al. 2012). Bei juvenilen männlichen SERT-KO-Ratten war das soziale Spielverhalten deutlich reduziert (Homberg et al. 2007b), und auch SERT-HET-Ratten zeigten im Erwachsenenalter soziale Defizite (Houwing et al. 2017). Ähnliche Befunde wiesen adulte SERT-KO-Ratten auf, die bei reziproken sozialen Interaktionen weniger Zeit im sozialen Kontakt verbringen, obwohl sie mehr Folgeverhalten zeigten (Golebiowska et al. 2019). Die Soziabilität war zwar nicht beeinträchtigt, es wurden aber Hinweise auf Defizite bei der sozialen Wiedererkennung gefunden (Golebiowska et al. 2019). Insgesamt scheint das Sozialverhalten bei SERT-KO-Ratten beeinträchtigt zu sein. Zudem gibt es Hinweise, dass mangelnde soziale Vorerfahrung durch soziale Isolation und Hierarchien einen Einfluss auf die 22-kHz USV-Produktion in stressvollen Situationen haben. Einzeln gehaltene Ratten vokalisieren weniger 22-kHz USV in Konfrontation mit einem milden, somatischen Stressor, während andere Verhaltensparameter wie Freezing und Defäkation keine Unterschiede zwischen einzeln und gruppengehaltenen Tieren aufweisen (Inagaki et al. 2005). Eine verringerte Emission von 22-kHz USV könnte also auch soziale Defizite widerspiegeln, und es wäre interessant zu sehen, ob die soziale Informationsverarbeitung durch SERT-Mangel beeinträchtigt wird, beispielsweise durch die Messung von Verhaltensreaktionen auf 22-kHz USV in Playback-Experimenten (Brudzynski und Chiu 1995; Fendt et al. 2018).

4.2.2.3 Genotypeffekt auf das USV-Spektrum

Die hier vorliegenden Befunde der genotypabhängigen Effekte auf 50- und 22-kHz USV weisen tendenziell in dieselbe Richtung wie die geschlechtsabhängige Verschiebung auf dem USV-Spektrum. Über den Einfluss des serotonergen Systems auf die Verschiebung der Rufmuster über diese Befundlage hinaus lässt sich lediglich spekulieren, da zu diesem Zeitpunkt – und nach aktuellem Wissensstand – keine vergleichbaren Untersuchungen vorliegen. Allerdings kann die Vermutung, dass es sich hier um genotypspezifische Effekte in der Resilienz gegenüber zunehmend aversiven Umgebungen handelt, die sich in einer verzögerten Verschiebung auf dem USV Spektrum verdeutlichen, nicht uneingeschränkt angenommen werden. Im Gegensatz zu den geschlechtsabhängig übereinstimmenden Befunden verschiedener Verhaltensindikatoren von Angst und Furcht, finden sich keine derartigen Zusammenhänge im SERT-KO-Modell. Vielmehr

ist hier das Gegenteil der Fall. Die genotypabhängigen Unterschiede in der 50-kHz USV-Produktion variieren zwischen den einzelnen Studien, während sich die Verringerung der 22-kHz USV als robust erweist. Hinzu kommen abweichenden Befunde anderer Verhaltensindikatoren von Angst und Furcht. Im Gegensatz zu den geschlechtsspezifisch geringen Angst- und Furchtmaßen der Weibchen, zeigen SERT-KO-Ratten mehr angstähnliches Verhalten im EPM und langsameres Extinktionslernen. Diese divergierenden Effekte des SERT-Mangels und somit der Zusammenhang von USV und anderen Verhaltensindikatoren bedarf demnach weiterer Klärung (siehe folgendes Kapitel 4.2.3).

4.2.3 Verhältnis von USV und Lokomotion

In allen hier vorliegenden Studien wird deutlich, dass USV-Emission und lokomotorische Inhibition nicht eindeutig deckungsgleich sind und somit wahrscheinlich unterschiedliche Facetten defensiven Verhaltens abbilden. Die berichteten Effekte wiesen meist in dieselbe Richtung, stehen gleichzeitig jedoch nicht immer in einem statistischen Zusammenhang:

1) Drei der fünf vermeintlich anxiogenen Substanzen aus Studie I gehen sowohl mit einer erhöhten Immobilität als auch mit einer verringerten 50-kHz USV-Produktion einher, ohne dass eine statistische Korrelation dieser Maße gefunden wurde.

2) In Studie II lösten alle verwendeten natürlichen und artifiziellen Geruchsproben Abwehrverhalten aus, allerdings auch hier in unterschiedlich starker Ausprägung und in einem statistisch nicht abbildbaren Zusammenhang; während Löwenurin und synthetisiertes TMT eine lokomotorisch basierte Vermeidungsreaktion in Form von reduziertem Kontakt mit der Geruchsprobe auswiesen, evozierten ausschließlich die natürlichen Geruchsproben von Fuchs- und Löwenurin 22-kHz USV. Die stark ausgeprägte lokomotorische Inhibition durch TMT in Abwesenheit jeglicher 22-kHz USV könnte die Diskussion um den Status des artifiziellen Geruchs als räuberischer oder lediglich schädlicher Stimulus weiter antreiben.

3) Auch die furchtinduzierte lokomotorische Inhibition und 22-kHz USV-Produktion der durchgeführten Furchtkonditionierungsparadigmen stehen nur in einem losen positiven Zusammenhang. Ratten emittierten 22-kHz Rufe zwar primär in Phasen der Immobilität, ein positiver statistischer Zusammenhang von 22-kHz USV und Immobilität wurde jedoch nur für einige Subgruppen der Untersuchung festgestellt (Studie III). Diese Diskrepanz zwischen furchtinduzierter Immobilität und 22-kHz USV-Emission könnte auf lediglich partiell

überlappende neuronale Schaltkreise zurückzuführen sein, die beide Komponenten der Furchtreaktion steuern. Tatsächlich wurden divergierende Bahnen aus BLA und CeA mit Immobilität bzw. 22-kHz USV-Emission in Verbindung gebracht. Es wurde gezeigt, dass neurotoxische Läsionen der BLA sowohl die Immobilität als auch die 22-kHz USV beeinträchtigen (Koo et al. 2004), während neurotoxische Läsionen des zentralen Kerns der Amygdala die Emission von 22-kHz USV stärker beeinträchtigen (Choi und Brown 2003). Interessanterweise wurde auch die Reaktion auf natürlichen Raubtiergeruch, jedoch nicht auf TMT, mit einer Aktivierung der BLA in Verbindung gebracht (Übersicht: Takahashi et al. 2005), während andere Überlegungen eher einen Einfluss des BNST im Ausdruck unkonditionierter Furcht gegenüber TMT postulieren (Fendt et al. 2003; Rosen 2004). Der Zusammenhang von 22-kHz USV, konditioniertem und unkonditioniertem Furchtverhalten bedarf demnach weiterer Klärung.

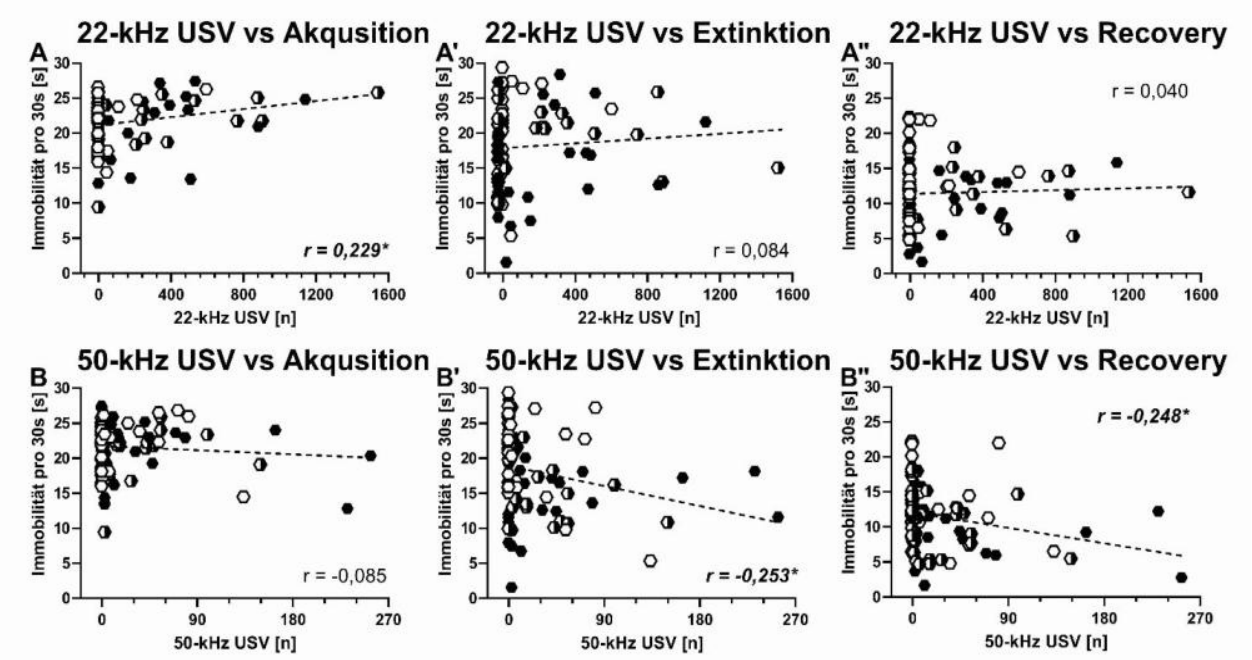
4) Die Einschätzung, dass Immobilität und 22-kHz USV-Emission zumindest teilweise unterschiedliche Aspekte der Furchtreaktion widerspiegeln, wird durch die Tatsache gestützt, dass sie während der Akquisitionsphase positiv korreliert waren, nicht jedoch während der Extinktion und Recovery, was auf eine geringe Vorhersagequalität der akuten 22-kHz USV Antwort für konditionierte Furcht hindeutet. Stattdessen ist die Performanz der Ratten während des Extinktionslernen und der Recoveryphase mit den zuvor 50-kHz USV assoziiert (siehe Abbildung 7). 22-kHz USV-Emission sind demnach mit anderen Maßen defensiven Verhaltens als Antwort auf akute Bedrohung assoziiert, während die Neigung 50-kHz USV zu produzieren mit der Geschwindigkeit und dem Erfolg des Inhibitionslernens zuvor konditionierter Reaktionen zusammenhängt.

Weitere Hinweise auf distinkten Effekte von Angst und Furcht auf 50- und 22-kHz USV-Produktion, und der partiellen Entkopplung von lokomotorischer Aktivität und USV-Emission liefert eine Studie zur vermeintlich anxiolytischen Wirkung des Neuropeptid S (NPS) (Kõiv et al. 2021). Einerseits zeigten sich anxiolytische Effekte von NPS im erhöhten Zero-Maze, die der geläufigen Annahme der durch NPS induzierten Anxiolyse entsprechen (Grund und Neumann 2019). Allerdings wurden wider Erwarten eine Vielzahl von 22-kHz USV detektiert. Verringerte Angst und erhöhte Furcht scheinen demnach teilweise unabhängige Phänomene zu sein. Die paradoxen Effekte von NPS auf Angst- und Furchtverhalten werden durch die fehlenden Auswirkungen auf die gemessenen 50-kHz USV jedoch weiterhin verkompliziert.

Anxiolyse wird im Tiermodell meist mit Hilfe von lokomotorischen Maßen wie Armeintritten, ortsabhängiger Verweildauer oder Ähnlichem festgestellt (Pellow et al. 1985; Pellow und File 1986). Den gängigen Maßen der Anxiolyse ist gemein, dass sie stark von der Grundrate der

lokomotorischen Aktivität des Tieres abhängen. Gemeinhin ist verminderte lokomotorische Aktivität mit negativ affektiven Zuständen assoziiert. Im Sinne des *Predatory Imminence Continuum* (Fanselow und Lester 1988) ist der angenommene lineare Zusammenhang zwischen verringerter Aktivität und negativem affektiven Zustand jedoch nicht haltbar. Sobald der negative affektive Zustand über Angst und Furcht hinaus in Panik übergeht, weicht die vorhergehende, furchtinduzierte Verhaltensstarre einer lokomotorisch stark ausgeprägten Fluchtreaktion [C*irca-strike Behavior*, Fanselow und Lester 1988]. Studie III hat gezeigt, dass die lokomotorische Aktivität der Ratten unmittelbar nach der elektrischen Stimulation am höchsten ist, wohingegen die Valenz der Situation keinesfalls weniger aversiv als an anderen Zeitpunkten der Testung sein kann. Die Tiere zeigen fluchtartiges Verhalten [*Escape-like Behavior,* Fanselow und Lester 1988]

Abbildung 7. Korrelation zwischen USV – Immobilität während der Furchtkonditionierung.



Anmerkung. Dauer der Immobilität während der Akquisition (A, B), Extinktion (A', B') und Recovery (A'', B'') im Verhältnis zu Anzahl der während der Akquisition emittierten 22-kHz USV (A, A', A'') und der während der Akquisition emittierten 50-kHz USV (B, B', B''). Abgebildet sind SERT-WT (schwarzer Diamant), SERT-HET (schwarz-weißer Diamant) und SERT-KO (weißer Diamant). *N* = 19 SERT-WT (15 weiblich, 14 männlich), 30 SERT-HET (15 weiblich, 15 männlich) und 28 SERT-KO (14 weiblich, 14 männlich). Daten sind individuelle Werte und Korrelationskoeffizienten. Die statistische Signifikanz ($p < 0.05$) des Korrelationskoeffizienten ist in ***Fett- und Kursivdruck*** angegeben.

In zusätzlichen Analysen wurde ein positiver Zusammenhang zwischen 22-kHz USV und Immobilität während der Akquisition festgestellt (22kHz-ACQ: r = 0.259, p = 0.015, A), dies war jedoch während Extinktion (22kHz-EXT: r = 0.130. p = 0.230, A') und Recovery (22kHz-REC: r = 0.087, p = 0.425, A'') nicht der Fall. Demgegenüber wurde kein Zusammenhang von 50-kHz USV und Immobilität während der Akquisition gefunden (50kHz-ACQ: r = -0.085, p = 0.435, B). Die während der Akquisition emittierten 50-kHz USV sagen jedoch die Immobilitätswerte der Extinktion (50kHz-EXT: r = -0.253, p = 0.018, B') und Recovery (50kHz-REC: r = -0.248, p = 0.021, B'') voraus. Tiere, die während der Akquisition mehr 50-kHz USV emittieren, zeigen weniger Immobilitätsverhalten in den darauffolgenden Phasen der Furchtkonditionierung.

auf Grund der unmittelbaren Nähe der akuten Bedrohung. Eine U-Funktion der lokomotorischen Aktivität in Abhängigkeit der Nähe zur Bedrohung/Gefahr ist also denkbar. Panikähnliche Zustände sind von vergleichbarer erhöhter Lokomotion gekennzeichnet wie appetitive Situationen, ohne dass sie eine entsprechend positive emotionale Valenz für die Ratte aufweisen. Erst unter Zuhilfenahme von USV lassen sich zuverlässige Interpretationen der situationsspezifischen Gegebenheiten treffen. Geht man im Hinblick auf die scheinbar paradoxen Befunde von Kõiv et al. (2021) davon aus, dass das Vorhandensein von 22-kHz USV in einem sonst vielfach mit 50-kHz USV assoziierten Setting wie dem Home Cage Test auf einen negativen affektiven Zustand hinweist, der von einer akuten Bedrohungssituation ausgeht und eine gesteigerte lokomotorische Aktivität durchaus panikähnliche Züge haben kann, scheint die hier berichtete Wirkung von NPS nicht mehr ausschließlich paradox. Vielmehr könnte es sich hier um eine über die Furcht hinausgehende Panikreaktion handeln, die von erhöhter lokomotorischer Aktivität und 22-kHz USV begleitet wird. Kõiv et al. (2021) betonen, dass die Gabe von NPS mit einer Stimulation der Hypothalamus-Hypophysen-Nebennierenrinden-Achse (HPA-Achse) assoziiert ist, die wiederum die Ausschüttung von Corticotropin-Releasing-Hormon (CRH) begünstigt (Smith et al. 2006). Die Applikation von CRH in stressvollen Situationen wiederum begünstigt die Emission von 22-kHz USV (Ise et al. 2008) und bestätigt die generelle Annahme, dass eine cholinerge Stimulation mit 22-kHz USV assoziiert ist (Brudzynski 1994; Brudzynski et al. 1991; Brudzynski 2015, 2014). Auf einer neuronalen Ebene wäre die Theorie einer gesteigerten panikähnlichen Reaktion, auf die das Vorhandensein negativ konnotierter 22-kHz USV hinweist, demnach durchaus haltbar.

Angst und Furcht als separate Phänomene zu betrachten, um gleichermaßen deren neuronale Korrelate untersuchen zu können, wurde bereits im Hinblick auf den furchtpotenzierten Schreckreflex [*Fear potentiated Startle*] untersucht (Davis 1998). Durch gezielte Manipulation der Stimuli lassen sich hier angstähnliche und furchtinduzierte Verhaltensweisen, zusammen mit ihren neuronalen Korrelaten voneinander trennen. In diesem Sinne erleichtert die Betrachtung der vorhandenen USV die Unterscheidung von Angst und Furcht, die auf lokomotorischer Ebene andernfalls Überlappungen aufweisen. Sowohl im EPM als auch in Furchtkonditionierungsparadigmen gilt eine Inhibition lokomotorischen Verhaltens als Hinweis auf angst- und furchtähnliche Zustände. Wird die lokomotorische Inhibition von einer verringerten 50-kHz USV-Emission begleitet, spräche das im Sinne des vorgeschlagenen Spektrums der USV für angstähnliches Verhalten; finden sich 22-kHz USV, ist eher von furchtinduziertem Verhalten auszugehen. Andere Untersuchungen zu Angst und Furchtverhalten sehen die Betrachtung der USV bereits als sinnvolle Ergänzung, da gängige Verhaltensmaße die

vorhandenen Effekte teilweise unterschätzen (Rojas-Carvajal und Brenes 2020); und bezeichnen USV als „direktes Fenster“ in die affektiven Zustände des Tieres (Kõiv et al. 2021; Panksepp 2005). Zudem wird angenommen, dass beispielsweise 22-kHz USV und furchtinduzierte Immobilität jeweils andere Funktionen erfüllen. Während 22-kHz USV oft im Zusammenhang mit dem Sozialgefüge und dessen (passiver) Alarmierung in Verbindung gebracht werden (Blanchard et al. 1991b; Blanchard et al. 1992; Brudzynski 2021), dient die lokomotorische Inhibition der eigenen Risikominimierung durch verminderte Detektionsmöglichkeit (Fanselow und Lester 1988; Perusini und Fanselow 2015). Die Betrachtung von USV trägt demnach zur besseren Validierung gängiger Modelle zu Angst- und Furchtverhalten bei. Mit der Hilfe von USV können eventuelle anxiogene und anxiolytische Effekte von zu untersuchenden Pharmaka eindeutiger spezifiziert werden. Die Vorhersage (prädiktive Validität) des Tiermodells gegenüber humaner Populationen nimmt zu und das Tiermodell kann gleichzeitig verfeinert werden (Konstruktvalidität) (Canteras und Blanchard 2008).

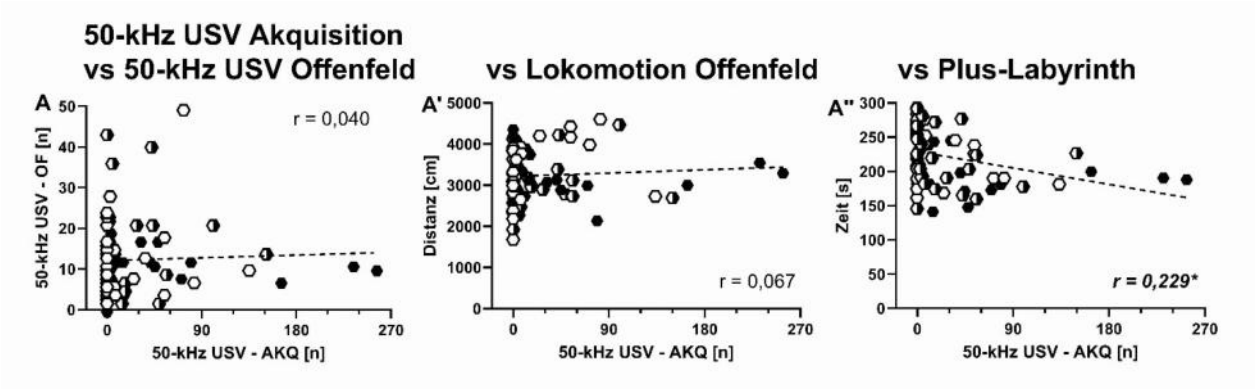
4.2.4 *Traits* – Charaktereigenschaften

In Studie IV lässt sich ein positiver Zusammenhang zwischen Ängstlichkeit [*Trait Anxiety*] – gemessen über das Verhalten im EPM – und Immobilitätsleveln während der Furchtkonditionierung feststellen, die bereits in anderen Untersuchungen gefunden wurden (Borta et al. 2006; Ilse et al. 2019). Interessanterweise sagt die Aktivität im EPM allerdings nicht die akute Reaktion auf elektrische Stimulation während der Furchtakquisition hervor, sondern lediglich die Performanz in Konfrontation mit dem CS während Extinktion und Recovery (Studie IV). Übereinstimmend damit wurden bereits Ängstlichkeit mit einem besseren Furchtgedächtnis (Fendt et al. 2021) und Defiziten bei Extinktion und deren Abruf berichtet (Muigg et al. 2008). Im Hinblick auf die USV-Produktion und angstähnliche Verhaltensweisen im EPM zeigt sich, dass die während der Furchtkonditionierung ausgestoßenen 22-kHz USV in keinem statistischen Zusammenhang mit der Verweildauer in den offenen Armen des EPM stehen, sondern lediglich mit den Immobilitätsleveln während der Akquisition korrelieren (Studie IV). Im Gegensatz dazu verbringen Tiere, die vermehrt in Phasen der Furchtkonditionierung 50-kHz USV vokalisieren, mehr Zeit in den offenen Bereichen des EPM (siehe ergänzende Analysen zu Studie IV - Abbildung 8). Auch hier zeigt sich demnach ein Zusammenhang zwischen einer erhöhten 50-kHz USV-

Emission und verringertem angstähnlichem Verhalten, während 22-kHz USV mit akuter Bedrohung durch elektrische Stimulation assoziiert sind.

Divergierende Zusammenhänge zwischen Rufverhalten und Immobilität in Abhängigkeit von Geschlecht und Genotyp sind ein weiterer Bestandteil des komplexen Verhältnisses von USV und Lokomotion. Während die Geschlechtsunterschiede der Immobilität und 22-kHz USV in Studie IV in dieselbe Richtung weisen, da weibliche Ratten sowohl weniger Immobilität als auch weniger 22-kHz USV zeigen; sind die Effekte des Genotyps inkonsistent. SERT-KO-Tiere emittierten weniger 22-kHz USV, behielten jedoch länger die konditionierte Reaktion der furchtinduzierten Immobilität bei. Vergleicht man die Zusammenhänge verschiedener Verhaltensweisen über mehrere Tests hinweg bezüglich stabiler Verhaltenstendenzen, finden sich Hinweise auf geschlechts- und genotypabhängige Charaktereigenschaften [*Traits*], die hier vor allem in Hinblick auf die Ängstlichkeit [*Trait Anxiety*] diskutiert werden sollen.

Abbildung 8. Korrelation zwischen 50-kHz USV – Verhalten in Offenfeld und Plus-Labyrinth.



Anzahl der 50-kHz USV während der Akquisition im Verhältnis zur Anzahl der 50-kHz USV der Offenfeldtestung (A), der lokomotorischen Aktivität während der Offenfeldtestung (A') und zur Verweildauer in den offenen Armen des EPM (A''). Abgebildet sind SERT-WT (schwarzer Diamant), SERT-HET (schwarz-weißer Diamant) und SERT-KO (weißer Diamant). *N* = 19 SERT WT (15 weiblich, 14 männlich), 30 SERT-HET (15 weiblich, 15 männlich) und 28 SERT-KO (14 weiblich, 14 männlich). Daten sind individuelle Werte und Korrelationskoeffizienten. Die statistische Signifikanz ($p < 0.05$) des Korrelationskoeffizienten ist in ***Fett- und Kursivdruck*** angegeben.

Zusätzliche Analysen haben ergeben, dass 50-kHz USV der Akquisitionsphase in keinem statistischen Zusammenhang mit den im Offenfeld emittierten 50-kHz USV stehen (50KHZ-50KHZ: r = 0.040. $p = 0.714$, A) oder der dort gezeigten lokomotorischen Aktivität (50kHz-DIS: r = 0.067, $p = 0.540$. A'). Allerdings wurde ein Zusammenhang zwischen 50-kHz USV während der Akquisition und Verweildauer auf den offenen Armen des erhöhten Plus-Labyrinths gefunden (50kHz-ZEIT: r = 0.229, $p = 0.033$, A''). Demnach verbringen 50-kHz vokalisierende Tiere mehr Zeit in den offenen Bereichen des erhöhten Plus-Labyrinths.

4.2.4.1 Ängstlichkeit und Geschlecht

Sowohl die lokomotorisch basierten Verhaltensmaße der Furchtkonditionierung und die furchtinduzierte 22-kHz USV-Produktion (Studie III und IV), als auch das Verhalten im EPM (Studie IV), deuten auf eine verminderte Ängstlichkeit weiblicher Ratten hin. Die Verhaltensprofile von Männchen und Weibchen über verschiedene Tests des angstähnlichen Verhaltens hinweg zeigen gemischte Geschlechtseffekte. Faktorenanalytische Betrachtungen mehrerer Tests mit großer Fallzahl wiesen auf ein verringertes angstähnliches Verhalten der Weibchen hin (Aguilar et al. 2003; Lopez-Aumatell et al. 2008). Hinzu kommen übereinstimmende, aber auch divergierende Einzelbefunde, die die Geschlechter vergleichen. Die Befunde zu lokomotionsbasierten Angsttests, wie beispielsweise die Messung der Ängstlichkeit an Hand der (fehlenden) Eintritte in das Zentrum des Offenfelds, die gemeinhin als Marker für Vermeidungsverhalten potenzieller Gefahrensituationen dient (Prut und Belzung 2003), sind gemischt. Während einige Autor:innen einen ängstlicheren Phänotyp der Weibchen in einem standardmäßigen Offenfeld von 40 x 40 cm Größe beschreiben (Bishnoi et al. 2021; Kosten et al. 2005) , finden sich solche Effekte in größeren Apparaturen, z.B. 54 x 80 cm, nicht (Scholl et al. 2019). Ortgebunde Maße, wie die An-/Abwesenheit im Zentrum der Testapparatur könnten demnach also von erheblichen Größenunterschieden adulter Männchen und Weibchen konfundiert sein. Oliveira Sergio et al. (2021) gehen weiterhin davon aus, dass eine Vielzahl der Testungen für angstähnliches Verhalten in Männchen entwickelt und unklare Validitäten für die Testung weiblicher Tiere aufweisen können. Unter „lebenswichtigen“ Bedingungen, wie der durch Neuheit unterdrückten Nahrungsaufnahme wiesen Weibchen durchaus ängstlichere Phänotypen auf, während andere Laborbedingungen eher auf ein geringes angstähnliches Verhalten deuten (Oliveira Sergio et al. 2021). Diese Beobachtungen spiegeln die bereits in Kapitel 4.2.1 beschriebenen divergierenden Befunde zur 22-kHz USV-Emission unter naturalistischen vs. artifiziellen Laborbedingungen wider. Festzuhalten bleibt demnach, dass die geschlechtsabhängigen Unterschiede der Ängstlichkeit zwar relativ robust zu sein scheinen, in diesem Zusammenhang USV jedoch nach wie vor nur selten gemessen werden. Ein Manko, das sich zu beheben versteht.

4.2.4.2 Ängstlichkeit und Genotyp

Im Gegensatz zu den gradlinigen Befunden zur geschlechtsabhängigen Ängstlichkeit, deuten die lokomotorisch basierten Verhaltensmaße der Furchtkonditionierung des EPM (Studie IV) für die

SERT-KO-Ratten in eine andere Richtung als deren furchtinduzierte 22-kHz USV-Produktion (Studie III und IV). In beiden Geschlechtern zeigte sich ein deutlicher Effekt auf angstähnliches Verhalten im EPM, welches bei SERT-KO-Ratten sichtlich erhöht war. Erhöhtes angstbezogenes Verhalten wurde durchgängig in früheren Studien beobachtet, in denen Paradigmen verwendet wurden, die geeignet sind, Auswirkungen auf die Angst aufzuzeigen (Olivier et al. 2008; Golebiowska et al. 2019; Johnson et al. 2019; Schipper et al. 2011; Sakakibara et al. 2014). Dies deutet darauf hin, dass die Veränderungen, die Ratten mit SERT-Mangel während der Furchtkonditionierung zeigen, zumindest teilweise durch eine höhere Ängstlichkeit bedingt sein können, die allerdings keine Entsprechung in einer vermehrten 22-kHz USV-Produktion findet.

4.2.4.3 Neuronale Korrelate der Ängstlichkeit

Bemerkenswerterweise können die generellen Geschlechtseffekte der Ängstlichkeit, beispielsweise im EPM durch die Manipulation des serotonergen Systems nivelliert werden. 5-HT selbst scheint demnach an diversen geschlechtsabhängigen Unterschieden beteiligt zu sein. Zuvor ängstlichere Männchen wiesen nach einer 5-HT-Depletion durch Hemmung der 5-HT-Synthese dieselben Verhaltensmaße auf wie Weibchen (Näslund et al. 2013; Näslund et al. 2015). 5-HT, Geschlecht und Ängstlichkeit hängen demnach in einer vielschichtigen Weise zusammen, sind aber untrennbar miteinander verbunden (Übersicht: Guimarães et al. 2010). Auch auf Ebene der neuronalen Strukturen finden sich Überlappungen geschlechtsspezifischer Charakteristika und serotonerger Transmission, die mit Ängstlichkeit in Verbindung gebracht wurden. Ein vielversprechender Kandidat hierfür ist der BNST, der bereits mit Angst in Verbindung gebracht wurde (Fanselow und Lester 1988; Fanselow und Ponnusamy 2008; Davis 1992, 1998). Der BNST ist bei Ratten geschlechtsdimorph (del Abril et al. 1987; Lebron-Milad und Milad 2012; Lebow und Chen 2016) und könnte somit das divergierende Ausgangsniveau der Ängstlichkeit in männlichen und weiblichen Ratten erklären. Außerdem spielt die serotonerge Transmission im BNST eine entscheidende Rolle für angst- und furchtähnliche Verhaltensweisen, da ein 5-HT-empfindlicher inhibitorischer Mikroschaltkreis des BNST Angst und aversives Lernen moduliert (Marcinkiewcz et al. 2016). Einerseits begünstigt die 5-HT Projektion der DRN zum BNST den Abruf konditionierter Furcht und verstärkt angstähnliches Verhalten im EPM (Marcinkiewcz et al. 2016). Andererseits übernimmt beispielsweise der 5-HT_{2C}-Rezeptor eine wichtige Funktion in der angstbezogenen Erregung des BNST (Marcinkiewcz et al. 2016). Die neuronalen Zielgebiete zukünftiger Untersuchung der Zusammenhänge von Geschlecht, serotonerger Transmission und Angst- bzw. Furchtverhalten sind demnach abgesteckt.

4.2.5 Klinische Implikationen

Tiermodelle sind entscheidend für das Verständnis klinischer Störungsbilder (Homberg et al. 2021) und helfen, die interindividuellen Mechanismen zu verstehen, die zu systematischen Unterschieden im Angst- und Furchtverhalten führen. So kann beispielsweise eine defizitäre Extinktionsfähigkeit, Kernkomponente der PTBS (Goswami et al. 2013), im Tier modelliert und somit eingehend untersucht werden (Milad und Quirk 2012; VanElzakker et al. 2014). Sowohl Extinktionsdefizite auf Grund frühkindlicher Traumatisierung (Xiong et al. 2014), PTBS-typische Hypervigilanz (Olszyński et al. 2021) oder persönlichkeitsabhängiges verstärktes Vermeidungslernen (Allen et al. 2019) sind nur einige Beispiele hierfür. Neben der Betrachtung situationsspezifischer Einflüsse, die die Konstitution von Angst und Furcht bedingen, sind in diesem Zusammenhang vor allem die biologischen Komponenten pathologischer Angst von besonderem Interesse.

Die Geschlechtsdimorphismen von Angst im Allgemeinen und Angststörungen im Speziellen sind im Humanbereich weitreichend bekannt, ihre neurologischen Grundlagen jedoch bis dato wenig erforscht (Bangasser und Cuarenta 2021; Day und Stevenson 2020; Lonsdorf und Merz 2017). Es gibt im Tiermodell jedoch schon Hinweise darauf, dass die neuronalen Grundlagen erfolgreicher Extinktion geschlechtsspezifische Unterschiede aufweisen und somit in der therapierelevanten Grundlagenforschung unbedingt beachtetet werden sollten (Gruene et al. 2015a; Gruene et al. 2015b). Zudem werden geschlechtsspezifische Unterschiede angstbezogener Störungsbilder zunehmend mit dem serotonergen System assoziiert (Songtachalert et al. 2018).

Wie bereits erwähnt, wurde das 5-HT-System im Tiermodell vielfach mit Angst und Furcht assoziiert (Guimarães et al. 2008; Bauer 2015; Deakin und Graeff 1991; Lowry und Hale 2010), und spielt beim Menschen eine Schlüsselrolle bei der Ätiologie von Angststörungen und beeinflusst zugleich die Wirksamkeit der Behandlung (Knuts et al. 2014; Lonsdorf et al. 2009; Gordon und Hen 2004; Guimarães et al. 2010). Es gibt Hinweise, dass der 5-HTTLPR-Polymorphismus das Risiko für die Entwicklung einer PTBS nach starker Traumaexposition erhöht (Gressier et al. 2013). Auf der anderen Seite beeinflusst der Polymorphismus des 5-HTTLPR die Stabilität inhibitorischer Lernprozesse (Wannemüller et al. 2018). Dementsprechend haben Studien gezeigt, dass in Abhängigkeit des 5-HTTLPR die Wirksamkeit der expositionsbasierten Therapie verringert ist (Bryant et al. 2010). Eine aktuelle Meta-Analyse findet jedoch keinen moderierenden Einfluss des 5-HTTLPR auf den Erfolg kognitiv-behavioraler Therapie anderer Angsterkrankungen (Schiele et al. 2021).

Die Amygdala als zentrale Komponente der neuronalen Schaltkreise, ist im Zusammenhang serotonerger Transmission besonders entscheidend für die Verarbeitung von emotionalen, insbesondere angst- und furchtbezogenen Reizen (Asan et al. 2013) und wurde ebenfalls als maßgebliche Struktur für PTBS benannt (Übersicht: Harnett 2020). Es gibt Hinweise, dass die emotionale Dysregulation bei PTBS unter anderem mit einer Überantwort der Amygdala auf (traumarelevante) Stimuli assoziiert ist (Shin et al. 2006; Fitzgerald et al. 2018; Giotakos 2020). Zudem wurden Unterschiede in der Furchtextinktion bei verschiedenen Spezies wiederholt mit der Aktivierung der Amygdala in Verbindung gebracht (Hariri et al. 2002; Furmark et al. 2004; Johnson et al. 2019; Shan et al. 2018).

Eine gezielte Untersuchung geschlechtsspezifischer, serotonerg orchestrierter Amygdala Aktivität in verschiedenen Stadien des Angst- und Furchtverhaltens ist unter Zuhilfenahme des sensiblen Maßes der USV in verschiedenen Situationen nur ein Aspekt, den es weiterhin zu untersuchen lohnt.

4.3 Limitationen und Ausblick

Obgleich der robusten Effekte auf die 50- und 22-kHz USV-Emission, sind die Befunde der Studie I und II vorerst nur auf einen Teil der Gesamtpopulation generalisierbar, da in diesen Untersuchungen ausschließlich männliche Ratten getestet wurden. Die Befundlage zu den geschlechtsabhängigen Unterschieden in etablierten Angst- und Furchtparadigmen – ebenso wie in Studie III und IV – verdeutlicht jedoch den Bedarf an einer weitergehenden Untersuchung unter Berücksichtigung des Geschlechts.

Während die Befunde über das Auftreten von 22-kHz USV-Emission (Studie II, III und IV) vergleichsweise unisono für ein furchtinduziertes Phänomen sprechen, ist die Verringerung der 50-kHz USV-Produktion in anxiogenen Kontexten (Studie I, III und IV) weitaus diffuser und kann meist nur im Vergleich mehrerer Kontexte untereinander beurteilt werden. Aus der Diskussion der vorliegenden Befunde zur angstinduzierten Verringerung der 50-kHz USV-Emission und dem aversionsabhängigen Übergang zur furchtinduzierten 22-kHz USV-Produktion wird deutlich, dass die intraindividuelle Vokalisationsneigung in verschiedenen Situationen über stabile Merkmalsausprägungen wie beispielweise *Trait Anxiety* Aufschluss geben kann. Zukünftige Untersuchungen im Sinne der Studien I, II und III würden dementsprechend von der Hinzunahme ergänzender Tests zur Erstellung eines detaillierten Verhaltensprofils profitieren.

Im direkten Vergleich der verwendeten Furchtkonditionierungsparadigmen der Studien III und IV wird deutlich, dass die Stärke biologischer Einflussfaktoren in Abhängigkeit des Designs variiert. Obgleich beide Paradigmen einander stark ähneln, sind sie nicht deckungsgleich und ziehen minimal divergierende Schlüsse über den Einfluss biologischer Faktoren wie Geschlecht und serotonerger Transmission. Während die Befunde zur USV-Emission in Großteilen nahezu identisch sind, weichen die lokomotionsbasierten Maße der beiden Studien voneinander ab. Eine detaillierte Auseinandersetzung mit den entscheidenden situationsspezifischen Faktoren innerhalb der Furchtkonditionierungsparadigmen, wie Intensität der verwendeten Stimuli, Kontextkonfiguration, Boden- und Deckeneffekte der gemessenen Verhaltensweisen könnte perspektivisch helfen, allgemeingültige Aussagen zum Einfluss biologischer Faktoren auf die Furchtkonditionierung zu treffen. Darüber hinaus sind die Schlussfolgerungen zu (Ultraschall-)Vokalisationsverhalten unter panikähnlichen Zuständen in Studie III und IV bis jetzt rein spekulativ. Eine detaillierte Differenzierung über das gesamte Verhaltensspektrum aversiver Situationen wäre wünschenswert. Auch die gegenteiligen Zusammenhänge von USV und lokomotionsbasiertem Verhalten in Abhängigkeit von Geschlecht und serotonerger Transmission in Studie IV konnten nicht abschließend geklärt werden. Über die neuronalen Marker geschlechts- und genotypabhängiger Zusammenhänge von USV, Immobilität, Angst und Furcht kann zu diesem Zeitpunkt nur spekuliert werden.

Abschließend bleibt zu erwähnen, dass die Ableitung emotionaler Zustände in Tiermodellen immer nur eine Annäherung über das gemessene Verhalten und die Zuschreibung homologer Zusammenhänge zwischen Mensch und Tier sein kann. Dennoch sind Tiermodelle eine wichtige Quelle für das tiefergehende Verständnis (pathologischen) Angst- und Furchtverhaltens. Auch wenn der Einfluss situationsspezifischer Faktoren in Abhängigkeit biologischer Determinanten nicht abschließend geklärt werden konnte, liefert die vorliegende Arbeit einen empirischen Beitrag zum Verständnis von Angst und Furcht im Tiermodell.

4.4 Fazit

Zur Beurteilung des emotionalen Zustands von Angst und Furcht in der Ratte als Modellorganismus ist die Betrachtung der emittierten USV unerlässlich. In Abhängigkeit situationsspezifischer Einflüsse, wie beispielsweise Neuheit, Beschaffenheit der Umgebung oder aversive Manipulation, lässt sich mit Hilfe des vorgestellten Spektrums der USV eine qualifizierte Aussage über das Vorhandensein von Angst oder Furcht treffen. Hierbei ist festzuhalten, dass ein

angstähnlicher Zustand, evoziert durch ungewisse, vage Situationen durch eine Verringerung der ausgestoßenen 50-kHz USV markiert wird, während durch spezifische Bedrohungen evozierte Furcht von 22-kHz USV begleitet ist. Mit Hilfe der Verortung situationsspezifischer USV können weiterhin biologische Einflüsse auf Angst- und Furchtverhalten systematisiert werden. Sowohl Geschlecht als auch serotonerge Transmission moderieren die Verortung situationsspezifischer USV, indem sowohl weibliche Ratten als auch SERT-KO-Tiere trotz zunehmender Aversivität de Situation die Emission von 50-kHz USV beibehalten und gleichwohl unter situationsspezifisch aversiven Bedingungen der Furchtkonditionierung weniger 22-kHz USV emittieren. Bemerkenswerterweise zeigen die Ausprägungen stabiler Verhaltensprofile wie Ängstlichkeit [*Trait Anxiety*] von Weibchen und SERT-KO-Tieren einen divergierenden Zusammenhang von lokomotionsbasiertem Angst- und Furchtverhalten und USV. Während die verschobene USV-Produktion der Weibchen mit einer gleichermaßen niedrigeren *Trait Anxiety* einhergeht, steht die verschobene USV-Produktion der SERT-KO-Tiere im Gegensatz zu deren erhöhten Angstmaßen. Es bleibt festzuhalten, dass die getrennte Betrachtung von Angst- und Furchtverhalten durchaus Beachtung verdient.

5 PUBLIKATIONEN

STUDIE I

Willadsen M, Best LM, Wöhr M, & Clarke PBS (2018). Effects of anxiogenic drugs on the emission of 22-and 50-kHz ultrasonic vocalizations in adult rats. *Psychopharmacology*, 235(8), 2435-2445.

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ORIGINAL INVESTIGATION

Effects of anxiogenic drugs on the emission of 22- and 50-kHz ultrasonic vocalizations in adult rats

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Abstract

Rationale Adult rat 22-kHz vocalizations are often associated with alarm or distress, whereas a subset of 50-kHz calls is preferentially emitted in response to amphetamine and other rewarding stimuli. Whether any 50-kHz calls reflect anxiety is unknown.

Objective To determine the effects of anxiogenic drugs on 50-kHz call rate and call subtype profile, in comparison with D-amphetamine.

Methods Adult male rats received systemic amphetamine (1 mg/kg) three times several days before testing. Ultrasonic vocalizations were then recorded after acute intraperitoneal injection of amphetamine or one of five anxiogenic drugs: yohimbine (2.5 mg/kg), *N*-methyl-*β*-carboline-3-carboxamide (FG 7142, 5 mg/kg), pentylenetetrazol (PTZ, 20 mg/kg), *m*-chlorophenylpiperazine (mCPP, 1 mg/kg), caffeine (25 mg/kg), or vehicle.

Results The duration of immobility was increased by FG 7142, PTZ, and mCPP; this measure was unchanged by yohimbine and reduced by the locomotor stimulant drugs amphetamine and caffeine. Conversely, the 50-kHz call rate was reduced by FG 7142, PTZ and mCPP, and increased by caffeine and amphetamine. Overall, the most common 50-kHz call subtypes were flat, trill, step-up, and complex. Consistent with previous reports, amphetamine increased the relative prevalence of trill calls while reducing the relative prevalence of flat calls. Yohimbine and caffeine reduced flat call prevalence, whereas mCPP reduced trill call prevalence. No other shifts in the call profile were observed, and no anxiogenic drug induced 22-kHz calls.

Conclusion Anxiogenic drugs, as a class, did not uniformly alter the 50-kHz call rate or subtype profile. Amphetamine-induced effects on 50-kHz call rate and profile do not reflect anxiety.

Keywords Ultrasonic vocalization · Anxiety · FG 7142 · *m*-Chlorophenylpiperazine (mCPP) · Anxiogenic · Amphetamine

Introduction

In the search for novel anxiety tests (Haller and Alicki 2012), rodent ultrasonic vocalizations (USVs) are of interest since they appear to convey information about emotional states (Brudzynski 2013; Burgdorf and Moskal 2010; Miczek et al. 1995; Wöhr and Schwarting 2010). Rats, in particular, emit USVs in three broad categories: infant 40 kHz, adult 22 kHz, and adult 50 kHz. To date, two of these three USV categories have been exploited as measures of anxiety: infant 40-kHz isolation calls that are triggered by maternal separation, and adult "distress/alarm" 22-kHz calls that are evoked by electric shock or shock-conditioned stimuli (Miczek et al. 1995; Sanchez 2003; Schwarting and Wöhr 2012; van der Poel and Miczek 1991).

Adult 50-kHz vocalizations, in contrast to 40- and 22-kHz calls, are spontaneously emitted in many behavioral contexts

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(Brudzynski 2013; Clarke and Wright 2014; Panksepp and Burgdorf 2010; Simola 2015; Wöhr and Schwarting 2010), but a possible association with anxiety has not been investigated extensively. The rate of 50-kHz call emission is reportedly inhibited by several anxiogenic conditions, including cat odor, repeated intermittent footshock, and bright illumination presented with or without an elevated platform (Ishiyama and Brecht 2016; Panksepp and Burgdorf 2010; Taylor et al. 2017). In contrast, 50-kHz call rate was decreased by neither yohimbine nor caffeine, even when given at doses that are typically anxiogenic (Mahler et al. 2013; Simola et al. 2016, Simola et al. 2010). A striking feature of 50-kHz calls is their high degree of heterogeneity, with 14 call subtypes identified to date (Wright et al. 2010), but it is unknown whether anxious rats preferentially emit any particular kind of 50-kHz call (Wright et al. 2010).

The main aim of the present study was therefore to establish whether one or more 50-kHz call subtypes, or possibly a change in the 50-kHz call rate, might serve as a marker for anxiety. To this end, we asked whether systemic administration of prototypical anxiogenic drugs produces a uniform and characteristic shift in the 50-kHz call profile. The five drugs selected for this purpose were yohimbine, FG 7142, pentylenetetrazol (PTZ), meta-chlorophenylpiperazine (mCPP), and caffeine. All five drugs are reported to be anxiogenic in human subjects and to produce anxiety-like behavior in rats, via diverse but incompletely identified receptor mechanisms (Table 1 and Supplementary Table 1). In particular, mCPP appears to act mostly via 5-HT2C receptor agonism (Gibson et al. 1994; Kennett et al. 1989), whereas FG 7142 and PTZ likely act via negative allosteric modulation of $GABA_A$ receptors (Evans and Lowry 2007; Huang et al. 2001). Mechanisms underlying yohimbine and caffeine anxiogenesis have yet to be identified, but it is unlikely that the main pharmacological targets of these drugs (α2 adrenergic receptors and adenosine receptors, respectively) play a significant role in rodents (Baldwin and File 1989; El Yacoubi et al. 2000; La Marca and Dunn 1994; Redfern and Williams 1995).

In the present study, we performed parallel tests with the psychostimulant D-amphetamine. This drug served in part as a positive control, since it reliably increases the 50-kHz call rate and predictably alters the call profile, i.e., the relative proportions of individual 50-kHz call subtypes (Wright et al. 2010). Specifically, amphetamine decreases the relative prevalence of the "flat" call subtype while promoting a narrowly defined "trill" call subtype (Wright et al. 2010). This particular call profile shift, which is also found with the euphorigens cocaine and morphine, has been proposed to reflect positive affect (Best et al. 2017; Pereira et al. 2014; Wright et al. 2012; Wright et al. 2010). In the present study, we tested the specificity of this claim, by investigating whether the same call profile shift is also associated with drug-induced anxiety.

Methods

Subjects

Subjects were 24 experimentally-naïve male Long-Evans rats (Charles River Laboratories, St. Constant, Quebec, Canada), weighing 310–360 g at the beginning of the experiment. Only male rats were used, for two reasons. First, all anxiogenic test drugs and doses have been extensively characterized in male but not female rats, with some evidence of sex differences either in baseline performance or anxiogenic effects of drugs (Haleem 1993; Hughes and Hancock 2016; Johnston and File 1991). Second, we wished to determine whether anxiogenic drugs would mimic the effects of amphetamine on 50-kHz call emission, which, to our knowledge, have been characterized in male rats only. The rats were housed three per cage (25 × 48 × 20 cm) in a temperature- and humidity-controlled colony room (19–20 °C, 50–60%) at the McGill University Animal Research Center. Home cage bedding consisted of laboratory-grade SaniChips™ (Harlan Laboratories, Indianapolis, IN). Rats were maintained on a reverse 12:12-h light/dark cycle with lights off at 0700 hours, and all testing was performed during the dark phase of the cycle. Food and water were available ad libitum except during testing. Rats were each handled once daily for 3 min, for 3 days prior to the habituation day. All procedures were approved by the McGill Animal Care Committee in accordance with the guidelines of the Canadian Council on Animal Care.

Test box

The test box comprised four vertical walls enclosing a square arena (61 × 62.5 cm). The walls were 53 cm high and made of wood composite coated by white plastic laminate. The enclosed floor area was covered with a layer of bedding (SaniChips™, as in the home cage) which was replaced between test sessions. The test box was lit by far-red (wavelength > 650 nm) illumination provided by two 40-W incandescent lights, each in combination with a Kodak GBX-2 safelight filter (Vistek, Toronto, ON, Canada) located 1.4 m above the floor. A video tracking system (EthoVision version 3.0, Noldus Information Technology, Leesburg, VA, USA) measured locomotor activity (expressed as the total distance moved) and immobility duration. The latter measure represented the total accumulated time spent with the animal exhibiting zero horizontal movement, as detected by the imaging system (set at five frames/s). Immobility duration served to approximate freezing behavior and was previously found to be correlated with aversive 22-kHz USV emission (Wöhr and Schwarting 2008).

Acquisition and acoustic analysis of ultrasonic vocalizations

Broadband recordings and acoustic analysis were performed essentially as detailed in our recent publications (e.g., Scardochio and Clarke 2013). Rats were recorded individually via an ultrasonic condenser microphone that was positioned 50 cm above the center of the test box and connected to an UltraSoundGate 416H data acquisition device (Avisoft Bioacoustics). The sampling rate was 250-kHz with 16-bit resolution. Spectrograms were generated by fast Fourier transform (512 points, 75% overlap, FlatTop window, 100% frame size) using Avisoft SASLab Pro (Version 5.2.07). All calls in a given session were manually selected from spectrograms by one individual (M.W.) and then verified by another (L.M.B.). Time-sampling was not used, i.e., all calls were analyzed in each test session. Fifty-kilohertz calls were categorized according to our 14-subtype scheme (Wright et al. 2010). Twenty-two-kilohertz calls were also identified, but rarely occurred.

Drugs

Drugs were as follows: caffeine and D-amphetamine sulfate (both from Sigma-Aldrich, Oakville, ON); FG 7142 (*N*-methyl-β-carboline-3-carboxamide), pentylenetetrazol (PTZ, 6,7,8,9-tetrahydro-5*H*-tetrazolo[1,5-*a*]azepine), mCPP (m-chlorophenylpiperazine HCl), and yohimbine HCl (all from Tocris Bioscience, Minneapolis, MN). Drugs were dissolved in sterile 0.9% saline, with the following two exceptions. Yohimbine was dissolved in distilled water, as it was insufficiently soluble in saline. FG 7142 was prepared as a suspension in a mixture of 5% (*v*/*v*) Tween-20 and 0.9% saline, and sonicated for 5 min. Immediately after preparation, all drugs were divided into aliquots and stored at − 20 °C until the day of use. All drugs were administered by intraperitoneal (IP) injection in a volume of 1 ml/kg. The control for each drug condition was the corresponding vehicle. All doses refer to the salt of the compound (except caffeine). Each drug was tested at a single dose, as follows: yohimbine 2.5 mg/kg, FG 7142 5 mg/kg, PTZ 20 mg/kg, mCPP 1 mg/kg, caffeine 25 mg/kg, and amphetamine 1 mg/kg. Doses of the anxiogenic drugs were based on published studies of anxiety-like behaviors (see Supplementary Table 1 for references) and were intended to produce sub-maximal effects. All doses were sub-convulsive, as reported for yohimbine (Cole et al. 1995), FG 7142 (Leidenheimer and Schechter 1988), PTZ (Eidman et al. 1990), mCPP (Cioli et al. 1984), and caffeine (Chu 1981).

Experimental overview and protocol

The experiment comprised the following stages: habituation in the test room (one session), amphetamine pre-tests (three sessions), and the anxiogenic drug testing block (12 sessions). On any given day, rats were taken in their home cages to the test room and left to settle for at least 20 min.

Habituation (one test day) Each rat ($n = 24$) was individually placed in the test cage for 20 min before being returned to its home cage.

Amphetamine pre-tests (three test days) Each rat received three 10-min test sessions in the test box, one session per day, with sessions spaced 2 days apart. Each session started 30 min after amphetamine injection. The aim of these sessions was to increase 50-kHz call rates (Scardochio and Clarke 2013; Wright et al. 2013).

Drug testing block (12 test days) Each rat received 12 test sessions and was tested under all drug conditions, as follows: yohimbine 2.5 mg/kg, FG 7142 5 mg/kg, PTZ 20 mg/kg, mCPP 1 mg/kg, caffeine 25 mg/kg, and amphetamine 1 mg/kg. Control tests were performed under the respective vehicle condition, i.e., water (for yohimbine), Tween-20/saline (for FG 7142), or saline. Each drug and vehicle condition was tested once, except for saline (twice) and amphetamine (three times). Test days were spaced 48 h apart, and the order of drug and vehicle testing was determined by two 12 × 12 Williams squares, i.e., counterbalancing for first-order carryover effects. The experimenter (M.W.) was blinded to treatment conditions. On a given day, rats were tested individually in the test box, for 10 min starting 30 min after a single injection of a drug or vehicle. At the start of each session, rats were placed facing the same corner.

Data analysis and statistics

Data were analyzed using Systat v11 software (SPSS, Chicago, IL), and figures were generated using Prism 4 (GraphPad Software, La Jolla, CA). The main dependent variables analyzed were as follows: duration of immobility, distance moved, 50-kHz call rate, and the percentages of flat and trill calls (i.e., the two most prevalent 50-kHz call subtypes). In addition, 50-kHz call profiles were defined by the proportional contributions of all 14 call subtypes (Wright et al. 2010). The 50-kHz USV and locomotor data were consistent across the two saline tests and across the three amphetamine tests; hence, these data sets were collapsed across sessions. One rat received the incorrect treatment on several occasions, through a coding error, and was excluded from all analyses. Therefore, all results are reported for $n = 23$ rats, except where noted. Two outliers, identified by Grubb's test (two-tailed $p < 0.01$), were excluded from parametric analyses (specifically: rat #21 immobility under FG 7142, $z = 4.23$, and rat #6 percentage of flat calls under PTZ, $z = 3.03$).

Drug effects were analyzed by comparing each drug condition with its corresponding vehicle control (i.e., saline, water, or Tween-20). For this purpose, paired *t* tests were used,

provided that the parametric test assumptions were met. Otherwise, Wilcoxon signed-ranks tests were used, i.e., to assess the effect of yohimbine on percent flat calls, and to test effects of all drugs on call rate data.

Exploratory correlational analyses were employed to explore possible relationships between distance moved, immobility duration, 50-kHz call rate, percentage of flat calls, and percent of trill calls. Here, Pearson correlations were calculated (exception 50-kHz call rate, Spearman correlations). Before analysis, the effects of the six drugs were isolated by subtracting the corresponding vehicle (control) values. In most cases, the sample size (n) was 23 rats, but some drugs (mCPP, PTZ, FG 7142, yohimbine) eliminated calling in certain animals so that the percentage of flat and trill calls could not be calculated; in such cases, n ranged between 14 and 20 rats.

In view of the large number of statistical tests performed, the two-tailed significance threshold was set at 1% throughout.

Results

Table 1 summarizes the main findings.

22-kHz and 50-kHz call rates Only one rat made any 22-kHz vocalizations, and these occurred in a single session under amphetamine (41 calls). Total 50-kHz call rates (i.e., number emitted per 10-min session) are shown in Figs. 1 and 2a. This measure was increased by caffeine and amphetamine, and reduced by PTZ and mCPP (Wilcoxon tests $Z = 3.12$–4.14, $p = 0.0018$–0.0001), with a similar inhibitory trend for FG 7142 ($Z = 2.39$, $p = 0.0169$). Yohimbine did not detectably alter the 50-kHz call rate.

Flat and trill call subtypes The relative prevalence (percentage) of flat and trill calls is shown in Fig. 2b, c. As expected, amphetamine increased the percentage of trill calls ($t_{22} = 3.23$, $p = 0.0038$) at the expense of flat calls ($t_{22} = 2.79$, $p = 0.0106$). Flat calls were also suppressed by yohimbine (Wilcoxon $Z = 3.22$, $p = 0.0013$) and caffeine ($t_{22} = 3.08$, $p = 0.0054$), but unlike amphetamine, neither drug significantly promoted trill calls (yohimbine $p = 0.0685$, caffeine $p = 0.2609$). Trill calls were inhibited by mCPP ($t_{13} = 3.68$, $p = 0.0028$).

50-kHz call profiles The proportion of all 14 50-kHz call subtypes is shown in Fig. 3 (see Supplementary Tables 2 and 3 for *absolute* data). In virtually all treatment conditions, the most prevalent 50-kHz call subtypes were complex, flat, step-up, and trill. No call subtypes, other than flat and trill calls, were significantly affected by any drug.

Locomotor activity and immobility Locomotor activity (i.e., distance moved) was inhibited by PTZ and mCPP, and

Table 1 Summary of results in relation to published anxiety-related studies

	Yohimbine	FG 7142	PTZ	mCPP	Caffeine	Amphetamine
Dose tested (IP)	2.5 mg/kg	5 mg/kg	20 mg/kg	1 mg/kg	25 mg/kg	1 mg/kg
Present study						
22-kHz call rate	–	–	–	–	–	–
50-kHz call rate	–	↓?	↓	↓	↑	↑
% flat	↓	–	–	–	↓	↓?
% trill	–	–	–	↓	–	↑
Immobility duration	–	↑	↑	↑	↓?	↓
Horizontal LMA	–	–	↓	↓	↑	↑
Anxiety-related measures in rats (published studies)						
Freezing or immobility	↑	↑	n/a	n/a	↑?	Mixed
EPM (time in open arms)	↓	↓	↓	↓	↓	Mixed
Social interaction	Unchanged or ↓	↓	↓	↓	↓	Mixed
Other measures (published studies)						
Anxiogenic (humans)	Yes	Yes	Yes	Yes	Yes	Mixed
Horizontal LMA (rats)	Unchanged or ↓	Unchanged or ↓	↓	Unchanged or ↓	↑ or ↓	↑
CPA (rats)	CPA or CPP	CPA or no effect	CPP	No effect	CPA	CPP

Stimulatory and inhibitory drug effects are shown by ↑ and ↓ symbols, respectively. An added question mark denotes a non-significant trend, whereas an en dash (i.e., "–") indicates no trend. Findings are also shown from representative published studies which used the same or similar doses; the corresponding literature references are given in Supplementary Table 1. In the rat EPM and social interaction tests, anxiety-like behavior is shown as "↓"

n/a no information appears available at relevant doses, *PTZ* pentylenetetrazol, *IP* intraperitoneal, *LMA* locomotor activity, *EPM* elevated plus maze, *CPA/CPP* conditioned place aversion/preference

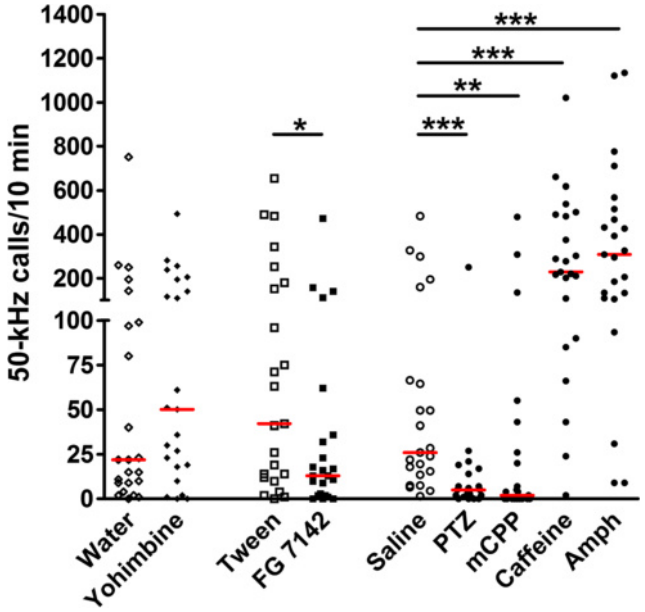

Fig. 1 Effects of anxiogenic drugs on the 50-kHz call rate. The *Y*-axis displays the number of calls made in the 10-min test session. Within a given drug condition, each data point represents an individual rat, and the horizontal bar shows the median. Each rat was tested under all drug conditions (see main text for details), and drugs are shown grouped with the respective vehicle condition, i.e., water, Tween-20/saline, or saline. Call rates were significantly increased by caffeine and amphetamine, and reduced by PTZ and mCPP. $*p = 0.02$ (i.e., trend), $**p < 0.01$, $***p < 0.001$ vs. vehicle control ($n = 23$ rats)

increased by caffeine and amphetamine ($t_{22} = 3.57$–7.93, $p = 0.0017$–0.0001; Fig. 2d). The corresponding percent changes were −25, −19, 18, and 35%, respectively, compared to vehicle control conditions. The duration of immobility was significantly increased by PTZ (by 88%, $t_{22} = 2.92$, $p = 0.0079$), mCPP (21%, $t_{22} = 2.98$, $p = 0.0070$), and FG 7142 (51%, $t_{21} = 3.18$, $p = 0.0045$), as shown in Fig. 2e. It was markedly *decreased* by amphetamine (by 70%, $t_{22} = 7.52$, $p = 0.0001$), with a similar trend for caffeine (35% decrease, $t_{22} = 2.81$, $p = 0.0101$), whereas yohimbine had no effect ($p = 0.6673$).

Correlational analyses Exploratory correlational analyses were used to explore possible relationships between the following variables: distance moved vs. immobility; percent flat vs. percent trill calls; and the two locomotor measures vs. 50-kHz call rate, percent flat, and percent trill. In this way, 48 correlation coefficients were generated (i.e., eight comparisons × six drugs), of which only four were statistically significant at the 1% level, as follows. Distance moved and immobility were negatively correlated ($p < 0.01$–0.001) with four individual drugs, i.e., yohimbine ($r = -0.7149$), PTZ ($r = -0.5886$), mCPP ($r = -0.5690$), and amphetamine ($r = -0.5698$), with similar trends ($p < 0.05$) for FG 7142 ($r = -0.4233$) and caffeine ($r = -0.4811$). The percentage of flat calls was not significantly correlated with

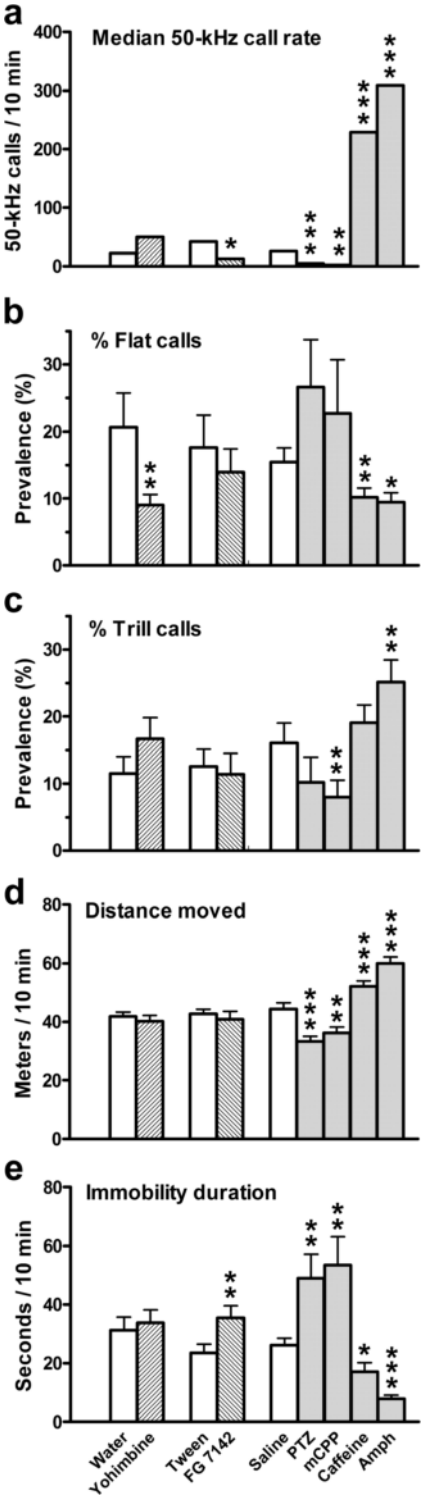

Fig. 2 Drug effects on 50-kHz vocalizations and video-based measures. *Y*-axes in panels **a**–**e** represent, respectively, median call rate (calls per 10-min test session), and mean ± SEM relative prevalence of flat and trill calls (expressed as a percentage of all 50-kHz calls), distance moved (m), and duration of immobility (s). Generally, $n = 23$ rats; however, calls were not emitted in certain individual sessions, hence for percentage flat and trill call measures, $n = 19$–23 rats except $n = 14$ rats for mCPP. $*p = 0.01$ (see text); $**p < 0.01$; $***p < 0.001$

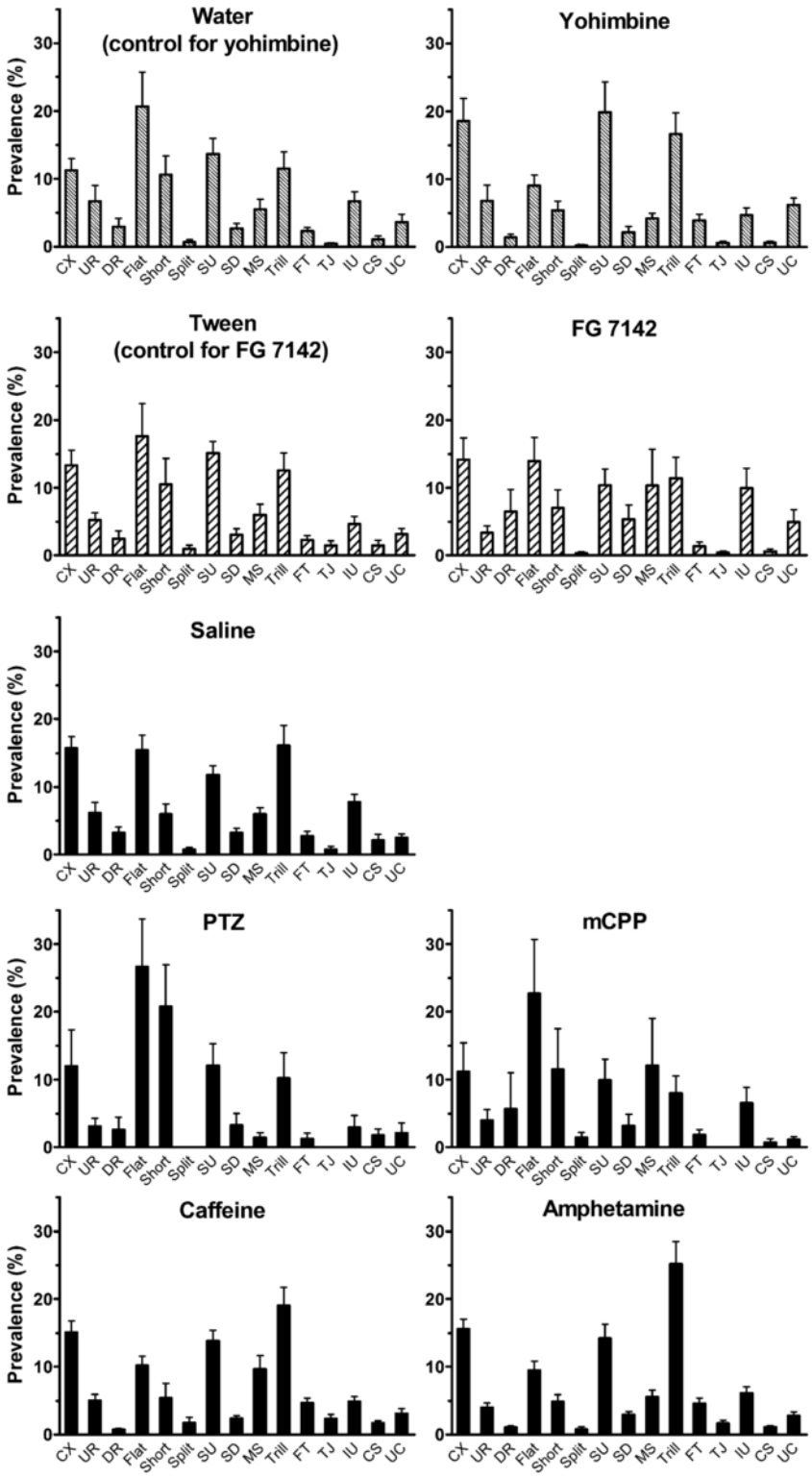

Fig. 3 Drug effects on 50-kHz call subtype profiles. *Y*-axes show the relative prevalence of each call subtype, expressed as the mean ± SEM percentage of all 50-kHz calls emitted. Call subtype abbreviations: CX complex, UR upward ramp, DR downward ramp, FL flat, SH short, SP split, SU step-up, SD step-down, MS multi-step, TR trill, FT flat-trill, TJ trill with jumps, IU inverted-U, CS composite, UC unclear. As explained in Fig. 2 legend, n = 19–23 rats in all conditions except for mCPP (n = 14). See Supplementary Tables 2 and 3 for absolute call rates

percent trill calls for any drug condition (r = − 0.4592 to + 0.1102). Similarly, neither of the two locomotor measures (distance moved and immobility duration) were significantly correlated with any of the three USV variables (r = − 0.4592 to + 0.5227). In particular, no significant correlation was found between the effects of any given drug on immobility and the 50-kHz call rate (Spearman rho = − 0.2743 to + 0.0845; Fig. 4).

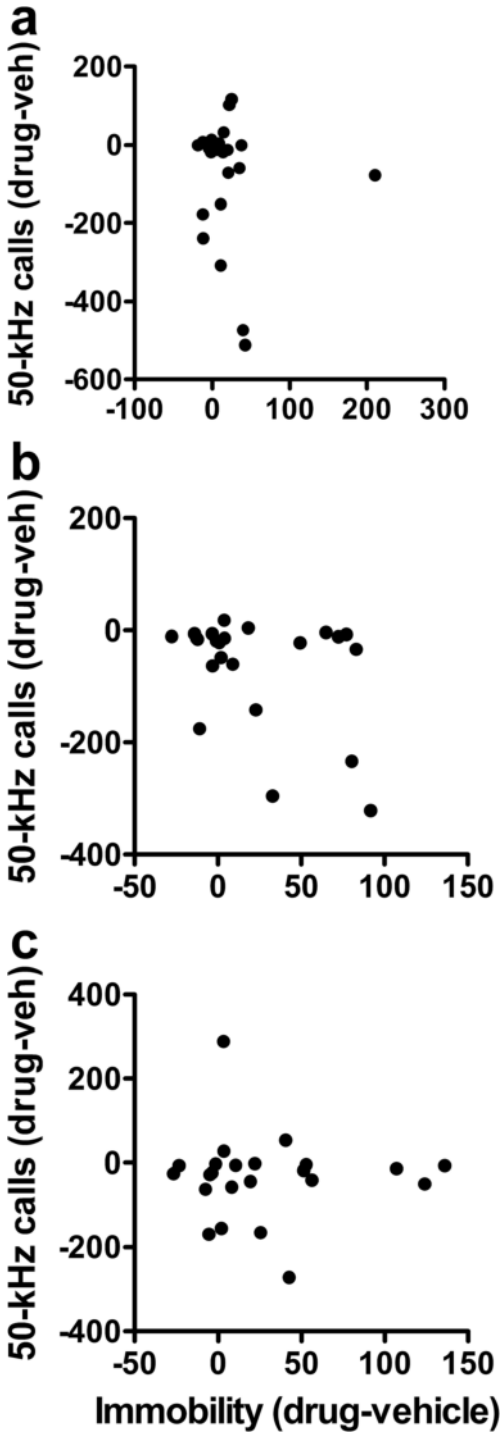

Fig. 4 Lack of relationship between drug effects on 50-kHz call rate and immobility duration, shown for FG 7142, PTZ, and mCPP (**a**–**c**, respectively). *Y*-axes show the number of calls per 10-min session, expressed as drug-minus-vehicle scores. *X*-axes show the vehicle-subtracted total time (s) per session spent immobile. Each point represents a single rat ($n = 23$)

Discussion

In the present study, we sought to determine whether drug-induced anxiety is accompanied by a characteristic shift in the 50-kHz call rate or subtype profile. Instead, the various anxiogenic drugs differed in terms of their effects on the 50-kHz call subtype prevalence as well as on the call rate. In addition, 22-kHz calls were notably sparse. Finally, no anxiogenic drug produced an amphetamine-like shift in the 50-kHz call profile.

Anxiogenic drug effects on 50-kHz vocalizations

The effects of anxiogenic drugs have received little attention in the context of 50-kHz vocalizations (see Introduction), with apparently no published reports concerning FG 7142, mCPP, or PTZ. All three drugs decreased the 50-kHz call rate. In contrast, caffeine markedly stimulated calling, confirming a previously reported trend (Simola et al. 2016; Simola et al. 2010), whereas yohimbine did not alter the 50-kHz call rate, also consistent with a previous report (Mahler et al. 2013).

The present study provides the first detailed analysis of individual 50-kHz call subtypes emitted by rats that are acutely challenged with anxiogenic drugs. The five anxiogenic test drugs exerted strikingly few effects on the relative prevalence of flat or trill call subtypes. Specifically, mCPP decreased the proportion of trill calls, whereas the proportion of flat calls was significantly decreased by yohimbine and caffeine (Table 1). Among anxiogenic drugs, it appears that only caffeine has previously been investigated with respect to 50-kHz call categories (Simola et al. 2010); in this earlier study, caffeine did not detectably change the relative prevalence of flat or trill calls, but these two categories were more broadly defined than in the present study.

Anxiety-like behavior vs. 50-kHz vocalizations

For each of the five anxiogenic drugs, we selected a dose and post-injection interval that has been reported to produce anxiety-like behavior in multiple behavioral assays (see Table 1 for examples, with corresponding literature citations in Supplementary Table 1). We measured immobility duration as an indicator of freezing-associated anxiety. Immobility was increased by three of the five test drugs (i.e., FG 7142, mCPP, and PTZ), but not by caffeine or yohimbine. Caffeine only marginally increased immobility or freezing at comparable doses in previous studies (Antoniou et al. 1998; Bhattacharya et al. 1997); quite possibly, immobility-related measures were contaminated by the locomotor stimulant effect of this drug. Yohimbine increased immobility in two previous reports (Bhattacharya et al. 1997; Park et al. 2001), but appears to perform inconsistently across behavioral assays, exerting anxiogenic-like effects in the elevated plus maze and social interaction test (e.g., Bhattacharya et al. 1997) but not in certain other test procedures (Baldwin et al. 1989; Jones et al. 2002; Molewijk et al. 1995).

In conclusion, only three of the five test drugs convincingly increased anxiety in the present study: FG 7142, mCPP, and PTZ. Of these, only mCPP significantly altered the prevalence of either flat or trill calls (Table 1), and no other call subtype appeared consistently affected. The only shared effect of these three drugs on 50-kHz call emission was a reduction in call rate. However, this shared inhibitory effect was not significantly correlated with drug-induced immobility and hence is unlikely to reflect anxiety.

Drug-induced aversion vs. 50-kHz vocalizations

At sufficiently high doses, most or all of the anxiogenic drugs that we tested produce not only anxiety but also extreme dysphoria in normal human subjects; such effects have been reported for FG 7142 (Dorow et al. 1983), mCPP (Charney et al. 1987; Murphy et al. 1989), PTZ (Good 1940; Rodin 1958), and yohimbine (Holmberg and Gershon 1961). However, strongly anxiogenic doses of caffeine and yohimbine do not always produce significant distress (Charney et al. 1983; Foltin and Fischman 1991; Lader 1969). Hence, the relationship between drug-induced anxiety and aversion is not always straightforward.

A similar complexity is seen in studies using adult rats, where anxiogenic drugs do not consistently produce conditioned place aversion (CPA), when given acutely at doses relevant to the present study. For example, neither FG 7142 nor mCPP produced a detectable CPA (Di Scala and Sandner 1989; Rocha et al. 1993), and mixed results were obtained with both PTZ (Bespalov 1996; Gauvin et al. 1991) and yohimbine (Chen et al. 2015; File 1986). Caffeine, in contrast, consistently produced a CPA (Brockwell et al. 1991; Patkina and Zvartau 1998; Steigerwald et al. 1988). The latter finding is potentially significant, since caffeine reduced the proportion of flat calls, an effect previously seen with *rewarding* drugs, i.e., amphetamine (Wright et al. 2010), cocaine (Wright et al. 2012), and morphine (Best et al. 2017). In future studies, therefore, it will be important to directly compare the effects of caffeine on 50-kHz call emission within the CPA procedure.

Amphetamine and 50-kHz vocalizations: relation to anxiogenic drugs

In the present study, acute amphetamine administration increased the 50-kHz call rate and increased the relative prevalence of 50-kHz trills, at the expense of flat calls, thus confirming previous studies (Wright et al. 2012; Wright et al. 2010). A shift in favor of the trill call subtype is also associated with cocaine and morphine administration, and has been proposed as an index of positive affect (Best et al. 2017; Wright et al. 2012, Wright et al. 2010). In the present study, the three most clearly anxiogenic drugs (i.e., FG 7142, mCPP, and PTZ) failed to exert amphetamine-like effects on any USV-related measure. This finding indicates that the effects of amphetamine on 50-kHz call emission do not reflect anxiogenic effects that are sometimes associated with this drug (Foltin and Fischman 1991); see Supplementary Table 1.

Drug effects on 22-kHz vocalizations

Adult rat 22-kHz "alarm/distress" calls have been widely used as a measure of anxiety or fear in adult rats, typically in conjunction with stressors such as air puffs, footshock, or acoustic startle (Sanchez 2003; Simola 2015). In this context, it has been proposed that 22-kHz calls reflect a "refractory, socially withdrawn or helpless state" (Sanchez 2003). In the present study, the anxiogenic drugs were tested in the absence of additional aversive stimuli, apart from the brief social isolation that occurred during the 10-min test session. Under these conditions, virtually, no 22-kHz calls were observed. These negative findings are consistent with previous studies using comparable doses of FG 7142 (Jelen et al. 2003), yohimbine (Mahler et al. 2013), PTZ (Portavella et al. 1993), and caffeine (Simola et al. 2010); mCPP does not appear to have been tested alone previously. While it remains possible that higher anxiogenic drug doses would have evoked 22-kHz vocalizations, as also found with higher-intensity electric footshock (Wöhr et al. 2005), the present findings nevertheless provide further evidence that anxiety per se is not generally sufficient to evoke 22-kHz vocalizations.

Study strengths and limitations

Study strengths include the detailed analysis of 50-kHz call subtypes, and the relatively powerful experimental design (i.e., 23 rats, repeated measures format). Limitations are as follows. First, testing rats individually was likely suboptimal, to the extent that ultrasonic vocalizations play a communicative role. However, while the effects of anxiogenic drugs would be worth reevaluating in a group setting, it is technically challenging to identify the specific animal emitting ultrasonic calls. Second, the open-field recordings yielded spectrograms that were somewhat less well-defined than those previously obtained in operant chambers (Wright et al. 2010), and hence acoustic properties such as bandwidth and frequency were not analyzed. Such an analysis would have been desirable, particularly since caffeine administration has been reported to affect these measures (Simola et al. 2010). Third, each drug was tested at only a single dose, and higher levels of anxiety might have produced a wider range of effects on USV emission. Nevertheless, all of our selected doses have been consistently reported to produce anxiety-like behavior in multiple studies, as documented in Supplemental Table 1, and all five anxiogenic drugs are associated with monotonic rather than inverted-U dose-response relationships (Bagdy et al.

2001; Baldwin and File 1989; Bhattacharya et al. 1997; Cole et al. 1995; File et al. 1988; File and Lister 1984; File et al. 1985; Wallis and Lal 1998). Fourth, each rat was exposed to several different drugs, and to repeated doses of amphetamine. While the experimental design was counterbalanced for carryover effects, this high degree of drug exposure may limit generalization to other studies; for example, with repeated drug administration, rats can become sensitized to amphetamine's acute effects on 50-kHz USV emission (Ahrens et al. 2009; Taracha et al. 2014). Lastly, our video tracking-based measure of anxiety (i.e., immobility duration) only approximates freezing behavior since the latter is commonly defined by a *crouched* immobile posture. However, the three test drugs which significantly increased immobility duration (FG 7142, mCPP, and PTZ) appear reliably anxiogenic at comparable doses.

Conclusions

In adult male rats, drug-induced anxiety is not sufficient to elicit 22-kHz calls but may be associated with reduced 50-kHz call emission when psychomotor stimulant effects are not present. Anxiogenic drugs, at moderate doses, do not selectively elicit any particular 50-kHz call subtype. Effects of amphetamine on 50-kHz call rate and subtype profile do not reflect anxiety.

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Compliance with ethical standards

All experiments comply with the current laws of Canada.

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Supplementary Table S1 Summary of results in relation to published anxiety-related studies

	Yohimbine	**FG 7142**	**PTZ**	**mCPP**	**Caffeine**	**Amphetamine**
Dose tested (IP)	2.5 mg/kg	5 mg/kg	20 mg/kg	1 mg/kg	25 mg/kg	1 mg/kg
Present study						
22-kHz call rate	-	-	-	-	-	-
50-kHz call rate	-	↓?	↓	↓	↑	↑
% flat	↓	-	-	-	↓	↓?
% trill	-	-	-	↓	-	↑
Immobility duration	-	↑	↑	↑	↓?	↓
Horizontal LMA	-	-	↓	↓	↑	↑
Anxiety-related measures in rats (published studies)						
	Yohimbine	**FG 7142**	**PTZ**	**mCPP**	**Caffeine**	**Amphetamine**
Freezing or immobility	↑[1,2]	↑[3]	n/a	n/a	↑?[1,4]	Mixed[4,5]
EPM (time in open arms)	↓[1,6-13]	↓[12,14-16]	↓[9,12,15-20] or ↓?[6]	↓[18,21-23]	↓[1,6,10] or ↑[17]	Mixed[6,24,25]
Social interaction	↓[1,20,26,27]	↓[15,20,28,29]	↓[15,30,31]	↓[32-34]	↓[1,35-37] **or** ↓?[10] or ↑[38]	Mixed[39,40]
Other measures (published studies)						
Anxiogenic (humans)	Yes[41,42]	Yes[43]	Yes[44,45]	Yes[46,47]	Yes[48]	Mixed[48,49]
Horizontal LMA (rats)	Unchanged[50] or ↓[1,6]	Unchanged[28] or ↓[51,52]	↓[6,19]	Unchanged or ↓[53,54]	↑[4,55,56] or ↓[1]	↑[4,5]
CPA (rats)	CPA[57] or CPP[50]	CPA or no effect[58]	CPP[59] or CPA[60]	No effect[61]	CPA[62-64]	CPP[65]

Notes: Stimulatory and inhibitory drug effects are shown by ↑ and ↓ symbols, respectively. An added question mark denotes a non-significant trend, whereas a dash (i.e. "-") indicates no trend. n/a - no information appears available at relevant doses. References in **bold font** are the most comparable to the present study, in terms of dose used, administration route, and testing interval. In the rat EPM and social interaction tests, anxiety-like behavior is shown as "↓". Abbreviations: PTZ, pentylenetetrazol; IP, intraperitoneal; LMA, locomotor activity; EPM, elevated plus maze; CPA/CPP, conditioned place aversion/preference.

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Supplementary Table S2
Absolute call rates for individual 50-kHz call subtypes: yohimbine and FG 7142 (and corresponding vehicle controls)

	CX	UR	DR	FL	SH	SP	SU	SD	MS	TR	FT	TJ	IU	CS	UC
Water (YOH vehicle)															
Median	3	1	0	2	3	0	3	1	1	3	0	0	1	0	1
Mean	10.7	8.5	1.3	12.6	3.4	2.7	15.6	1.8	3.5	12.9	4.2	0.5	8.8	0.7	2.0
Std. Error	4.2	4.7	0.4	5.1	0.8	2.0	6.8	0.5	1.3	5.8	2.0	0.2	4.8	0.3	0.6
Minimum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Maximum	90	106	7	85	15	44	155	9	27	117	44	3	109	5	10
Yohimbine (YOH)															
Median	9	2	0	4	3	0	8	1	1	5	1	0	2	0	3
Mean	14.2	5.2	1.3	11.1	6.1	0.4	20.5	1.7	5.0	16.0	4.9	1.4	4.6	0.8	6.3
Std. Error	4.2	1.8	0.4	4.1	3.3	0.4	6.8	0.4	1.4	4.1	1.7	0.9	1.1	0.3	1.8
Minimum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Maximum	96	34	7	80	77	9	142	6	19	68	29	17	18	5	34
Tween (FG 7142 vehicle)															
Median	7	2	1	5	3	0	13	1	2	3	0	0	2	0	1
Mean	14.2	8.0	1.4	22.6	4.3	4.7	24.6	3.2	8.9	16.2	5.5	2.0	10.1	2.1	4.3
Std. Error	3.9	3.1	0.5	10.5	1.1	3.2	8.1	1.3	2.8	5.4	2.0	1.0	4.0	1.1	1.9
Minimum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Maximum	74	54	10	223	19	69	123	28	48	115	34	16	62	26	43
FG 7142															
Median	2	0	1	2	1	0	1	0	0	0	0	0	1	0	0
Mean	5.9	3.7	1.0	6.7	1.9	0.6	8.4	1.1	2.0	10.3	2.4	0.1	3.7	0.3	2.0
Std. Error	2.0	2.5	0.3	2.4	0.7	0.4	4.8	0.4	0.9	5.0	1.7	0.1	1.7	0.2	0.9
Minimum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Maximum	30	57	6	46	13	7	109	7	19	103	38	1	36	3	20

Notes:
Values represent the absolute number of calls emitted per 10-min session for each call subtype. These data tended to be positively skewed, such that mean values were generally larger than the corresponding medians. n=23 rats, repeated measures design. Call subtype abbreviations: CX complex, UR upward ramp, DR downward ramp, FL flat, SH short, SP split, SU step-up, SD step-down, MS multi-step, TR trill, FT flat-trill, TJ trill with jumps, IU inverted-U, CS composite, UC unclear. The "miscellaneous" (MI) call subtype was entirely absent, hence not shown.

Supplementary Table S3

Absolute call rates for individual 50-kHz call subtypes: pentylenetetrazole, mCPP, caffeine and amphetamine (and saline controls)

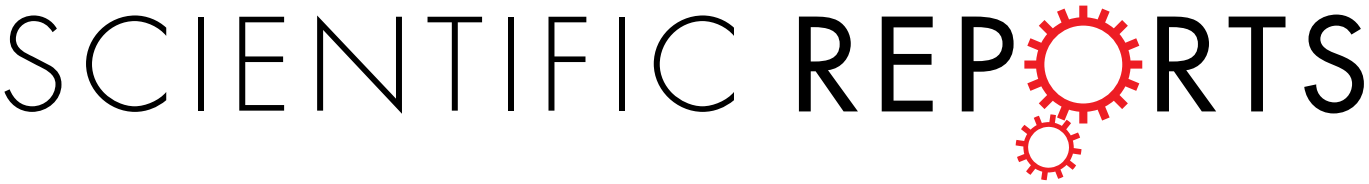
	CX	UR	DR	FL	SH	SP	SU	SD	MS	TR	FT	TJ	IU	CS	UC
Saline (vehicle)															
Median	5	1	1	2.5	1.5	0	3	0.5	1	5.5	0.5	0	1	0.5	0.5
Mean	10.0	8.3	1.3	12.5	4.3	2.0	12.2	2.6	5.4	12.0	2.7	0.5	6.2	1.0	2.1
Std. Error	2.7	3.8	0.3	4.7	1.5	1.0	4.8	0.9	2.0	4.2	1.4	0.3	2.0	0.3	0.8
Minimum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Maximum	45.5	73	6.5	92.5	26.5	21	90.5	15	35.5	94.5	32	5.5	26.5	5.5	15.5
Pentylenetetrazole															
Median	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Mean	1.4	1.9	0.3	2.7	1.2	0.0	4.3	0.3	0.6	2.2	0.7	0.0	0.8	0.3	0.5
Std. Error	0.6	1.6	0.2	1.4	0.4	0.0	3.5	0.1	0.4	1.2	0.7	0.0	0.6	0.1	0.4
Minimum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Maximum	11	38	4	33	6	0	81	2	8	27	15	1	13	2	9
mCPP															
Median	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mean	4.0	3.7	0.6	9.2	2.1	2.3	9.7	1.2	2.8	5.8	3.0	0.0	2.9	0.5	1.1
Std. Error	1.8	2.6	0.3	4.2	0.9	1.7	5.2	0.5	1.4	3.2	2.0	0.0	1.5	0.3	0.6
Minimum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Maximum	36	60	7	69	19	39	96	11	29	58	43	1	33	5	13
Caffeine															
Median	44	10	1	25	7	0	30	7	19	42	8	4	9	4	4
Mean	41.1	19.8	2.2	32.9	8.2	10.5	49.9	7.5	22.2	53.1	20.6	8.0	16.4	6.2	7.5
Std. Error	6.0	6.1	0.6	7.3	1.4	6.1	10.3	1.3	3.5	11.0	6.9	2.9	4.4	1.6	2.1
Minimum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Maximum	106	136	9	147	28	127	161	24	52	183	143	62	82	28	33.5
Amphetamine															
Median	31.0	6.3	2.0	26.0	8.3	0.3	29.7	7.3	14.0	56.0	9.7	1.3	11.3	1.3	7.7
Mean	48.0	18.5	3.5	38.7	11.5	5.5	51.0	10.4	18.8	98.7	26.2	5.9	19.9	4.0	7.6
Std. Error	8.9	6.7	0.9	7.7	2.0	3.2	11.4	2.6	4.6	22.6	9.7	1.9	4.2	1.2	1.3
Minimum	1.3	0.0	0.0	0.0	0.3	0.0	0.7	0.0	0.0	0.3	0.0	0.0	0.3	0.0	0.0
Maximum	145.7	146.0	20.3	124.0	31.7	64.7	212.3	44.3	102.7	441.0	173.0	37.0	71.0	22.0	17.7

Notes:

Values represent the absolute number of calls emitted per 10-min session for each call subtype. These data tended to be positively skewed, such that mean values were generally larger than the corresponding medians. n=23 rats, repeated measures design. Call subtype abbreviations: CX complex, UR upward ramp, DR downward ramp, FL flat, SH short, SP split, SU step-up, SD step-down, MS multi-step, TR trill, FT flat-trill, TJ trill with jumps, IU inverted-U, CS composite, UC unclear. The "miscellaneous" (MI) call subtype was entirely absent, hence not shown. Data for saline and amphetamine conditions were pooled across two and three sessions, respectively.

STUDIE II

Fendt M, Brosch M, Wernecke KE, **Willadsen M**, & Wöhr M (2018). Predator odour but not TMT induces 22-kHz ultrasonic vocalizations in rats that lead to defensive behaviours in conspecifics upon replay. *Scientific reports*, 8(1), 1-11.



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Predator odour but not TMT induces 22-kHz ultrasonic vocalizations in rats that lead to defensive behaviours in conspecifics upon replay

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Predator odours induce defensive behaviour in prey animals such as rats. The present study investigated (1) whether laboratory rats exposed to predator odours emit 22-kHz calls which may have an alarming function and (2) whether playback of such calls induces behavioural changes in conspecifics. For this, Sprague-Dawley rats were exposed to samples of fox and lion urine, as well as to the synthetic predator odour TMT. Despite that all odours induced defensive behaviour, only predator urine samples but not TMT were able to induce 22-kHz calls in a few rats. In a second experiment, naive rats were exposed to playback presentations of the 22-kHz calls recorded in the first experiment, as well as to phase-scrambled and frequency-shifted control stimuli. Low intensity playback presentations led to a reduction of locomotor activity during the presentation of the 22-kHz calls but not of the control stimuli. This effect was less specific under high intensity conditions. Taken together the present findings show that natural predator odours are able to induce emission of 22-kHz calls in rats and support the hypothesis that these calls have an alarming function.

For small animals like rodents, defence against predatory threat is a fundamental requirement of life[1] for often an encounter with a predator ends fatally for the prey[2]. To help to avoid and/or to survive encounters with a predator, rodents – as well as other prey animals – developed very efficient anti-predatory defence strategies, such as freezing[3]. As proposed in Fanselow's predatory imminence continuum theory[4], the defensive strategy is dependent on the perceived threat. In rats, only foraging and mating behaviour is typically changed at low risk conditions (pre-encounter stage), whereas at higher risk conditions (post-encounter stage) alterations in freezing or avoidance behaviour can be observed[5–7]. In this stage, also alarm signals can be emitted to warn conspecifics of the presence of predatory threat[8,9]. At the highest level of predatory imminence (circa-strike stage), jumping, fighting, and biting is expressed[4].

Notably, many of these defensive behaviours are not only elicited by the appearance of predators but also by stimuli predicting a predator such as predator odors[10,11]. In rats, the neuroethology of predator odour-induced defensive behaviours has been extensively studied during the last decades[12–14]. In most studies, the rats were exposed to cat odour, i.e. collars worn by cats[15,16] or blocks or cloths placed in a cat's bed or rubbed on the cat's body[17,18]. However, also odours of other potential predators such as ferrets, minks, foxes, bobcats, wolves, cougars, coyote, and lions were efficient in inducing defensive behaviors[19–23]. Several studies were able to identify single molecules derived from predator odours which are believed to be key components of the odour

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that triggers defensive behaviours, such as 2,5-dihydro-2,4,5-trimethylthiazoline (TMT) from fox feces[24–26] or 2-phenyl-ethylamine from carnivore urine[23].

A very prominent defensive behaviour displayed by rats is the emission of 22-kHz ultrasonic vocalizations[27–29]. These 22-kHz calls are usually relatively long, i.e. between 300 and 3,000 ms, and have a mean peak frequency of approximately 22 kHz, as indicated by their name. Since these calls are emitted in aversive situations, they are believed to reflect a negative emotional state[28], in order to transmit a potential threat to conspecifics[29], and/or to warn conspecifics about a potential threat[27]. In many studies, these calls were induced by foot shocks, air puffs, startle stimuli, or drug withdrawal[27–29]. Another approach to elicit 22-kHz calls is to expose rats to a conditioned stimulus (CS), usually a tone, which was previously paired with an unconditioned stimulus (US), typically a foot shock[30]. Surprisingly, there are only very few reports on 22-kHz calls induced by exposure to predators or to predator odours. As shown in the early 1990s by the Blanchards and colleagues[31–33], exposing rats to a cat efficiently induced the emission of 22-kHz calls. To the best of our knowledge, there is only one published study investigating whether exposure to predator odours can induce 22-kHz calls. In this study, rat lines selectively bred for high and low 50-kHz ultrasonic vocalizations were used[34], a call type typically occurring in appetitive situations and fulfilling a pro-social function as social contact calls[28,29]. Exposure to a worn cat collar induced low rates of 22-kHz calls in the line selectively bred for high but not low 50-kHz calls, while a substantial number of 22-kHz calls was seen in the random control line[34]. However, there were no odour-free control conditions in this experiment, as well as no detailed analysis of the recorded calls.

The aim of the present study was to characterize the effects of predator odour exposure on the emission of 22-kHz calls in male Sprague-Dawley rats. In our first experiment, rats were exposed to water (control odour), fox urine, lion urine, as well as TMT, with the aim of evaluating which of these odours induce defensive behaviours. The acoustic parameters of the recorded 22-kHz calls were analysed in detail. Our second experiment tested whether the playback of lion urine-induced-22-kHz calls leads to defensive behaviours in experimentally naive rats. To test specificity, fear CS-induced 22-kHz calls were also presented. Finally, to model differences in threat imminence, 22-kHz calls were presented with low and high sound intensity.

Materials and Methods

Ethical approval. All experiments were conducted in accordance with the European regulations for animal experiments (2010/63/EU) and approved by the local authorities (University of Magdeburg: Az. 42505-2-1172; University of Marburg: Az. MR 20/35 Nr. 19/2014).

Experiment 1 (University of Magdeburg): Predator odour-induced defensive behaviour and ultrasonic vocalizations. Animals and housing: Testing was carried out using 19 experimentally naive male (2–3 months old) Sprague-Dawley rats. Rats were bred and reared at the local animal facility (original breeding stock: Taconic, Denmark). They were housed in groups of 5–6 animals in standard Macrolon Type IV cages (58 cm × 33 cm × 20 cm) with water and standard lab chow (Altromin, Lage, Germany) available ad libitum. Cages were kept in temperature- and humidity-controlled rooms (22 ± 2 °C, 50–55%) with a 12:12 h light/dark cycle (lights on at 6:00 am). All behavioural tests were conducted during the light phase between 10:00 am and 5:00 pm.

Odour samples: 2,3,5-trimethyl-3-thiazoline (TMT) was purchased from PheroTech (Delta, Canada) and fox urine from Maine Outdoor Solutions Inc. (Hermon, ME, USA). Lion urine was obtained from the Zoo of Magdeburg, Germany. All urine samples were aliquoted into 1 ml portions and stored at −18 °C until usage. As a control odour, tap water was used.

Experimental setup and procedure: Testing took place in a standard Macrolon Type III cage (37.5 cm × 22.0 cm × 15.5 cm) covered by an acrylic transparent lid and placed under a fume hood (illumination: ~40 lux). The odour samples were presented in a glass bowl (4 cm diameter, 2.5 cm height), placed, and fixed in the middle of one short side of the test cage. On the opposite side, an ultrasound microphone (for details see below) was positioned outside the test cage next to a hole (diameter: 1.5 cm, height: 6 cm).

On the first day, each rat was singly placed into the test cage for 10 min (without odour sample) to familiarize the rats with the test cage. On the following four days, the odour exposure sessions were performed. First, the odour sample (1 ml of water, fox urine, lion urine, or 5 µl of TMT, respectively) was put into the glass bowl. Second, the experimental rat was gently positioned in the middle of the test cage. Third, the test cage was covered with a transparent acrylic plate to prevent diffusion of the odour through the fume hood. Test duration was 10 min. Each rat was tested once per day and four times in total, with the order of odour samples being counterbalanced within and across days. The test cage was thoroughly cleaned with soapy water after each test and ventilated with clean air.

Recording and analysis of predator odour-induced behavioural changes: Behaviour of the animals was recorded via a video camera mounted above the test cage. Computerized tracking software (EthoVision XT, Version 10, Noldus Information Technologies, Wageningen, The Netherlands) was used to automatically analyse the following behaviours: (a) immobility behaviour (EthoVision software: 2% immobility threshold, averaged over 5 samples), (b) distance travelled, and (c) time spent in odour and no odour area (1/3 of the test cage close and far away from the odour sample, respectively). Furthermore, (d) nose contacts of the animals with the odour sample (number and duration) were manually scored.

Recording and analysis of ultrasonic vocalization: The UltraSoundGate system from Avisoft Bioacustics (Berlin, Germany) was used for recording and analysing ultrasonic signals. For recording, an ultrasound condenser microphone (CM16/CMPA) sensitive to frequencies of 15–180 kHz (flat frequency response between 25 and 140 kHz; ± 6 dB) was used which was connected to a laptop via an USB audio device (UltraSoundGate 116 H). Acoustic data were recorded by AviSoft Recorder USGH software (version 4.2) using a sampling rate of 250,000 Hz in 16-bit format and a recording range of 0–125 kHz.

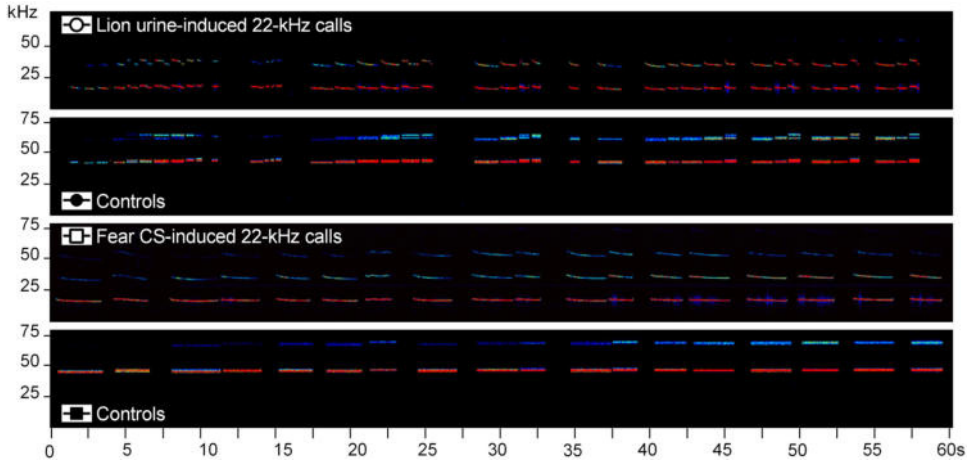

Figure 1. Spectrograms depicting the calls used for playback. The colours code the intensity of the calls (intensity correlates with warmness of the colour).

For offline analysis of the acoustic data, SASLab Pro software (version 5.2) was used. After a fast Fourier transformation (512 FFT length, 100% frame, Hamming window and 75% time window overlap), high resolution spectrograms were produced with a frequency resolution of 488 Hz and a time resolution of 0.512 ms. Onset and offset of the recorded 22 kHz calls were manually marked by a person who was not aware of the experimental condition, and the following parameters were determined and calculated for each single odour exposure session: latency of the first call, number of calls, number of bouts, mean calls per bout, mean call duration, and mean peak frequency. A bout was defined as a call, or a number of calls, separated from other calls by intervals longer than 320 ms[35].

Experiment 2: Playback of predator urine-induced 22-kHz calls (University of Marburg). Animals and housing: Testing was carried out using 20 experimentally naive male (2–4 months old) Sprague-Dawley rats. Rats were bred and reared at the local animal facility (original breeding stock: Charles River, Germany). They were housed in groups of 3–6 animals in standard Macrolon Type IV cages with high stainless steel covers (58 cm × 33 cm × 20 cm). Water and standard lab chow (Altromin, Lage, Germany) was available ad libitum. Cages were kept in temperature- and humidity-controlled rooms (22 ± 2 °C, 40–70%) with a 12:12 h light/dark cycle (lights on at 6:00 am). All behavioural tests were conducted during the light phase between 8:00 am and 5:00 pm.

Experimental setup and procedure: To test whether lion urine-induced 22-kHz calls induce defensive behaviours in experimentally naive rats, a modified playback protocol previously established was applied[36]. Defensive behaviour was assessed on an elevated radial eight-arm maze (arms: 40.5 × 9.8 cm) under dim red light (~10 lux) conditions[37].

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. Acoustic stimulus presentation was monitored using two ultrasonic condenser microphones (CM16, Avisoft Bioacoustics) placed next to the loudspeakers.

Four acoustic stimuli were used. This included (I) natural lion urine-induced 22-kHz calls (Fig. 1, first panel) and (II) phase-scrambled and frequency-shifted lion urine-induced 22-kHz controls (Fig. 1, second panel), with the latter serving as a time- and amplitude-matched acoustic stimulus control. To test specificity, this also included (III) natural fear CS-induced 22-kHz calls (Fig. 1, third panel) and (IV) phase-scrambled and frequency-shifted fear CS-induced 22-kHz controls (Fig. 1, forth panel), with the latter again serving as a time- and amplitude-matched acoustic stimulus control. Natural sequences of lion urine-induced and CS-induced 22-kHz calls were chosen to best reflect acoustic features typical for lion urine-induced and CS-induced 22-kHz calls, respectively, under conditions of good signal-to-noise ratios, yet with similar total calling times. Natural lion urine-induced 22-kHz calls and natural fear CS-induced 22-kHz calls were recorded in Magdeburg.

Natural lion urine-induced 22-kHz calls: The natural lion urine-induced 22-kHz calls were recorded from a male Sprague-Dawley rat exposed to lion urine, as described above. The acoustic stimulus contained n = 44 22-kHz calls (total calling time: 37.23 s). Average acoustic call parameters were as follows (mean ± SEM): call duration: 0.84 ± 0.06 s; peak frequency: 19.42 ± 0.11 kHz; downward slope: −0.43 ± 0.12 kHz.

Phase-scrambled and frequency-shifted lion urine-induced 22-kHz controls: The phase-scrambled and frequency-shifted 22-kHz controls were generated with SASLab Pro (Version 4.2, Avisoft Bioacoustics). Specifically, each given 22-kHz call in the original natural lion urine-induced 22-kHz stimulus was first phase-scrambled, i.e. the phase of the original signal was replaced by a random phase. The resulting signal exhibiting the original average power spectrum, but its waveform being a random noise signal, was then shifted up in frequency by 25 kHz, i.e. clearly out of the frequency range 22-kHz calls typically occur[28,29]. The acoustic stimulus contained n = 44 phase-scrambled and frequency-shifted 22-kHz calls (total calling time: 37.23 s).

Average acoustic call parameters were as follows (mean ± SEM): call duration: 0.84 ± 0.06 s; peak frequency: 44.42 ± 0.11 kHz; downward slope: 0.00 ± 0.00 kHz.

Natural fear CS-induced 22-kHz calls: The natural CS-induced 22-kHz calls were recorded from a male Sprague-Dawley rat exposed to five fear CS, a 10 kHz tone, in a retention test of a fear conditioning experiment, one day after a fear conditioning session with six pairings of the CS with a 0.8 mA foot shock. The acoustic stimulus contained n = 18 22-kHz calls (total calling time: 43.53 s). Average acoustic call parameters were as follows (mean ± SEM): call duration: 2.42 ± 0.11 s; peak frequency: 21.87 ± 0.13 kHz; downward slope: −3.26 ± 3.08 kHz.

Phase-scrambled and frequency-shifted fear CS-induced 22-kHz controls: The phase-scrambled and frequency-shifted 22-kHz controls were generated with SASLab Pro (Version 4.2, Avisoft Bioacoustics), as described for the phase-scrambled and frequency-shifted lion urine-induced 22-kHz controls. The acoustic stimulus contained n = 18 phase-scrambled and frequency-shifted 22-kHz calls (total calling time: 43.53 s). Average acoustic call parameters were as follows (mean ± SEM): call duration: 2.42 ± 0.11 s; peak frequency: 46.87 ± 0.13 kHz; downward slope: 0.00 ± 0.00 kHz.

All 20 rats were individually exposed to all four acoustic stimuli in counter-balanced order in two subsequent playback sessions separated by two to three weeks. Each session started with an initial 5 min habituation period. Then, the subject rat was exposed to 1 min playback presentations of natural lion urine-induced or CS-induced 22-kHz calls and the respective phase-scrambled and frequency-shifted 22-kHz control separated by a 10 min inter-stimulus interval, followed by a 5 min post-stimulus period.

To model differences in the imminence of threat, all four acoustic stimuli were presented with low and high sound intensity. In a first run with two subsequent sessions (low intensity playback), 22-kHz calls were presented with 40–50 dB SPL. In a second run with two subsequent session two to three weeks later (high intensity playback), 22-kHz calls were presented with 70–80 dB SPL.

Behaviour was monitored by a video camera (Panasonic WV-BP 330/GE, Hamburg, Germany) mounted centrally above the arena. Computerized tracking software (EthoVision XT, Version 10, Noldus Information Technologies, Wageningen, The Netherlands) was used to analyse locomotor activity (distance travelled).

Descriptive and analytical statistics. Behavioural data are expressed as means ± standard errors of the mean (SEM), whereas acoustic data are shown as whisker box plots. Statistical analyses were performed with GraphPad Prism (version 6.00, GraphPad Software Inc., La Jolla, USA). Data were checked for normal distribution (D'Agostino and Pearson omnibus normality test). Odour or playback effects were analysed by analysis of variance (ANOVA) using odour, area, stimulus, time, and phase as within-subject factors. A $p < 0.05$ was considered statistically significant.

Data availability statement. The datasets generated and/or analysed during the current study are available from the corresponding authors on reasonable request.

Results

Experiment 1: Predator odour-induced defensive behaviour and ultrasonic vocalizations. *Defensive behaviours.* Our analysis focused on behaviours known to be affected by predator urine samples or TMT[10,25]. Exposure to water was regarded as control condition, i.e. behaviour during exposure to the predator urine samples or TMT was compared with the behaviour during water exposure in post-hoc comparisons.

As shown in Fig. 2A, immobility of the rats was only increased during exposure to TMT [ANOVA: $F_{3,16} = 5.78$, $p = 0.005$; post-hoc Dunnett's test: $t_{18} = 4.21$, $p = 0.002$] but not during exposure to samples of fox and lion urine [$t_{18} = 0.14$, $p = 0.99$ and $t_{18} = 1.58$, $p = 0.29$, respectively]. Furthermore, there was a trend for odour effects on distance travelled (data not shown) [ANOVA: $F_{3,16} = 2.73$, $p = 0.07$].

Notably, the contact time with the odour samples was strongly affected by the odours [ANOVA: $F_{3,16} = 15.14$, $p < 0.0001$] (Fig. 2B). Post-hoc comparisons revealed a significant decrease of contact time with lion urine samples [$t_{18} = 5.13$, $p = 0.0002$] and TMT [$t_{18} = 5.77$, $p < 0.0001$] but not with fox urine samples [$t_{18} = 1.09$, $p = 0.57$].

Very similar results were obtained if the times spent in the area close to the odour samples (odour area) and the time spent in the area far from the odour sample (no odour area) were compared [ANOVA: $F_{1,36} = 4.94$; $p = 0.03$] (Fig. 2C). Post-hoc comparisons showed significantly different area times with lion urine samples [Fisher's LSD test: $t_{18} = 2.59$, $p = 0.01$] and TMT [$t_{18} = 2.57$, $p = 0.01$], indicating that these odour samples were avoided. This was not observed with water [$t_{18} = 0.26$, $p = 0.79$] or fox urine samples [$t_{18} = 0.76$, $p = 0.45$].

Ultrasonic vocalizations. Figure 3 shows the number of rats emitting 22-kHz calls during testing in different odour exposure conditions. None of the tested rats emitted 22-kHz calls during exposure to water or to TMT. Exposure to the predator samples induced 22-kHz calls in some animals [Chi-square test: $\chi^2 = 9.21$, $df = 3$, $p = 0.03$] (Fig. 3A). Whereas only one rat emitted 22-kHz calls in response to fox urine, four of the 19 rats emitted 22-kHz calls during exposure to lion urine (Fig. 3A). The median latency of the first 22-kHz call was 224 s [range: 72–345 s] (Fig. 3B), the median number of 22-kHz calls was 75 [range: 16–154] (Fig. 3C), the median number of bouts was 29 [range: 8–35] (Fig. 3D), and the median total time spent calling 80 s (range: 15–87 s; Fig. 3E).

We further analysed whether animals that emitted 22 kHz calls express more defensive behaviour than those that did not emit calls. Since only the exposure to lion urine lead to a decent number of vocalizing animals, the analysis was restricted to this condition. Vocalizing and non-vocalizing animals did not differ regarding immobility or contacts with the samples [t-tests: $ts < 0.51$, $p > 0.61$] (Fig. 3F,G). However, vocalizing rats spent less time in the odour area than in the no odour area [Sidak's multiple comparison: $t_3 = 2.57$, $p = 0.03$], an effect which was not observed in non-vocalizing rats [$t_{14} = 1.30$, $p = 0.36$; ANOVA: factor area: $F_{1,34} = 8.29$, $p = 0.007$; factor group: $F_{1,34} = 0.006$, $p = 0.94$; interaction: $F_{1,34} = 2.84$, $p = 0.10$] (Fig. 3I).

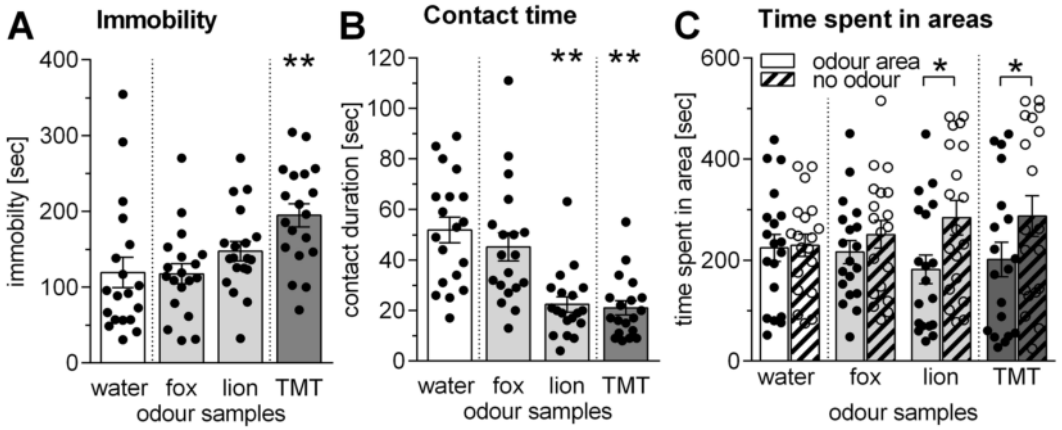

Figure 2. Exposure to predator odour samples induced defensive behaviour in rats. Using a repeated-measure design, rats (n = 19) were exposed to samples of water (control condition), fox urine, lion urine, and TMT. (**A**) Immobility, (**B**) contact time with the odour sample, and (**C**) time spent in the area close to the odour sample (odour area) or far from the odour sample (no odour) was measured and is depicted as means + SEMs. Only exposure to TMT increased immobility of the rats. Both lion urine samples and TMT were significantly less contacted than control samples and rats avoided the area close to these odours. Fox urine samples did not induce behavioural changes in this paradigm. *$p < 0.05$, **$p < 0.01$, post-hoc comparison with water or as indicated, after ANOVA.

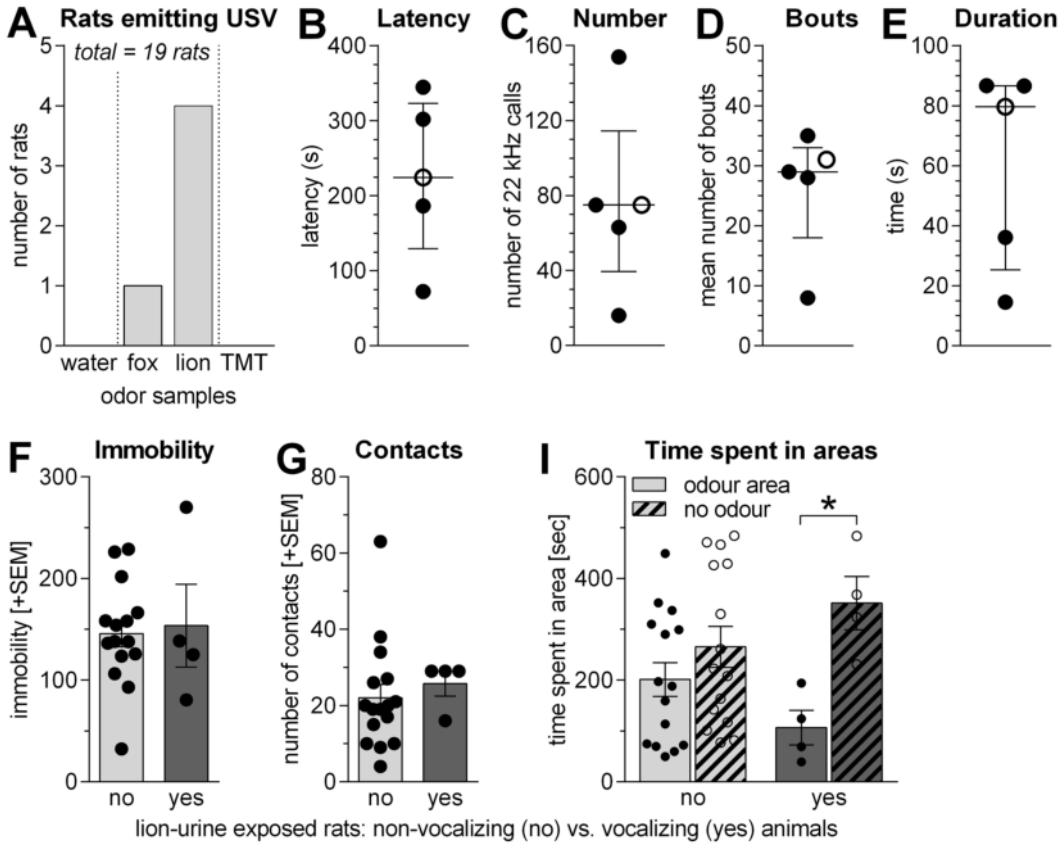

Figure 3. Exposure to predator urine samples induced 22-kHz calls. (**A**) Number of rats emitting 22-kHz calls during exposure to the different odour samples. (**B–E**) Scatter plots depicting the latency, total numbers, numbers of bouts, and total calling time of 22-kH calls for the individual vocalizing rats (open circle = exposure to fox urine; filled circle = exposure to lion urine). The horizontal lines indicate the medians and the interquartile ranges. (**F–I**) Defensive behaviour of non-vocalizing (no) vs. vocalizing (yes) lion urine-exposed rats. The 22-kHz call emitting rats expressed similar immobility (**F**) and contacts with the odour sample (**G**) but more avoidance behaviour (**I**) than the non-vocalizing animals. *$p < 0.05$, Sidak's multiple comparison, as indicated, after significant effects in ANOVA.

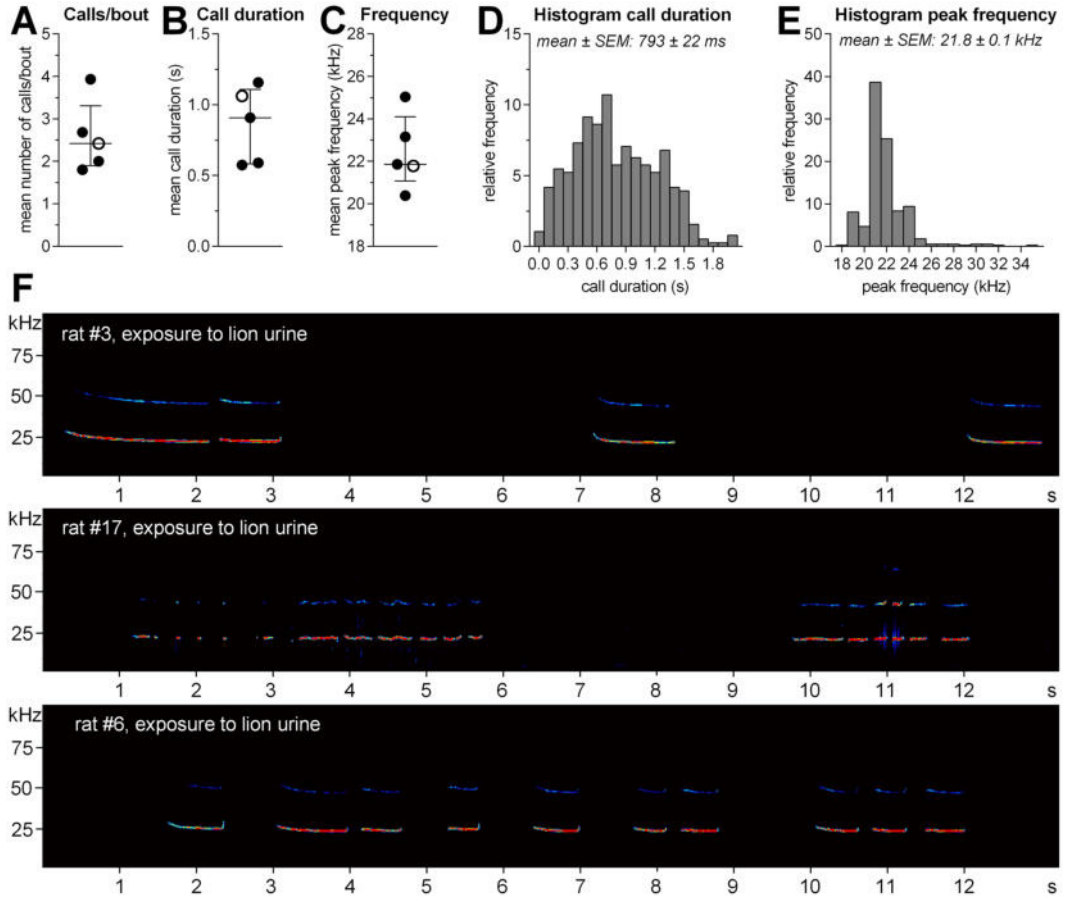

Figure 4. Acoustic parameters of 22-kHz calls induced by exposure to predator urine samples. (**A**) Mean number of calls per bout, (**B**) mean call duration, and (**C**) mean peak frequency of the calls. The horizontal lines indicate the medians and the interquartile ranges (open circle = exposure to fox urine; filled circle = exposure to lion urine). Histograms depicting the distribution of call duration (**D**) and peak frequency (**E**) of all calls recorded during exposure to the different predator urine samples (n = 383), bin widths: 0.2 s and 1 kHz, respectively. (**F**) Exemplary spectrograms showing examples of the recorded 22-kHz calls from three different rats. The colours code the intensity of the calls (intensity correlates with warmness of the colour).

Figure 4 depicts selected acoustic parameters of all recorded 22-kHz calls (n = 383), as well as some exemplary spectrograms of 22-kHz calls from different animals. Note the large variation in the duration of the recorded 22-kHz calls (Fig. 4D–F).

Experiment 2: Playback of predator urine-induced 22-kHz calls. In this experiment we tested whether playback of lion urine-induced 22-kHz calls (Fig. 1) leads to behavioural changes in naive rats. As control condition, rats were also exposed to playback presentations of 22-kHz calls recorded in a retention test of a fear conditioning experiment (Fig. 1). Rats were individually exposed to all four acoustic stimuli in two subsequent playback sessions. In each session, the rat was exposed to playback presentations of natural lion urine-induced 22-kHz calls (Fig. 1, first panel) or CS-induced 22-kHz calls (Fig. 1, third panel) and the respective phase-scrambled and frequency-shifted 22-kHz control (Fig. 1, second and fourth panel). To model differences in the imminence of threat, all four acoustic stimuli were presented in a first run with low sound intensity (40–50 dB SPL) and then in a second run with high sound intensity (70–80 dB SPL).

The behaviour most robustly affected by the playback of 22 kHz calls in the present study was distance travelled on the eight-arm maze (Fig. 5A–D). For each of the playback condition, the time course in 1-min blocks (left panels) and the means of the different phases (before, during, and after stimulus playback; right panels) are shown. Notably, playback of lion urine-induced 22-kHz calls with a low intensity but not the respective control induced an reduction of locomotor activity [ANOVA: interaction stimulus x time: $F_{10,190} = 2.55$, $p = 0.007$; time: $F_{10,190} = 3.74$, $p = 0.0001$; stimulus: $F_{1,19} = 0.03$, $p = 0.86$] (Fig. 5A). This reduction was only observed during stimulus presentation [post-hoc Sidak's comparisons for each minute: $t = 3.56$, $p = 0.005$ for stimulus phase; ts < 2.01, ps > 0.40 for all other minutes]. Analysis of the different phases of the test (right panel) supported the previous analysis, i.e. there was a reduction of locomotor activity during presentation of the lion urine-induced 22-kHz calls but not during presentation of the respective control [ANOVA: interaction stimulus x time: $F_{2,38} = 9.97$, $p = 0.003$; phase: $F_{2,38} = 15.83$, $p < 0.0001$; stimulus: $F_{1,19} = 0.79$, $p = 0.39$]. Again, post-hoc Sidak's comparisons showed significant differences during stimulus presentation [$t = 4.71$, $p < 0.0001$] but not in the pre- or

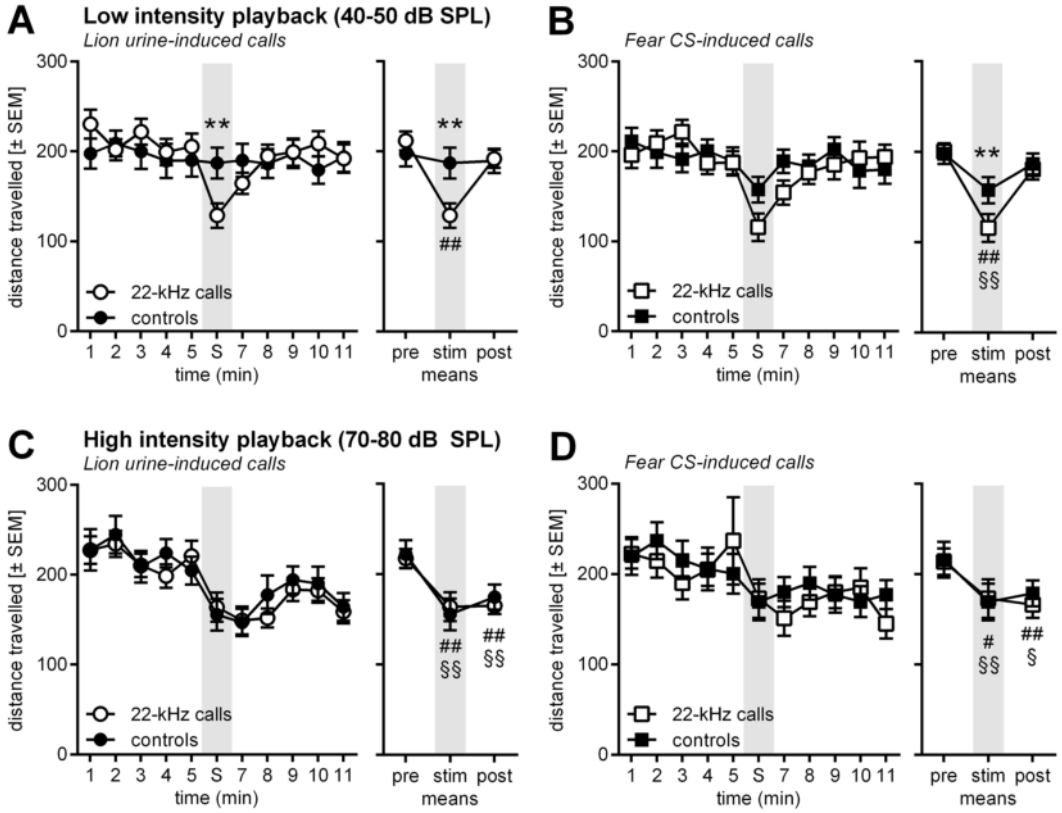

Figure 5. Low intensity playback presentations of lion urine-induced 22-kHz calls or fear CS-induced 22-kHz calls but not of their respective controls led to a reduction in locomotor activity on a radial maze. High intensity playback presentations unspecifically reduced locomotor activity during and after playback. Line diagrams depicting the mean locomotor activity (±SEM) of the rats in 1-min blocks (left panels; S or stim as well as the shaded area indicate the minute of playback presentation) or in the different phases of the experiment (right panel; pre, before playback; stim, during playback; post, after playback presentation). The rats were exposed to playback presentations of low intensity lion urine-induced 22-kHz calls and their respective phase-scrambled and frequency-shifted controls (**A**), low intensity fear CS-induced 22-kHz calls and the respective controls (**B**), high intensity lion urine-induced 22-kHz calls and the respective controls (**C**), and high intensity fear CS-induced 22-kHz calls and the respective controls (**D**). $^{**}p < 0.01$ (post-hoc Sidak's comparisons 22-kHz calls vs. control), $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ (post-hoc Sidak's comparisons vs. pre-phase; only 22-kHz calls), $^{\S}p < 0.05$, $^{\S\S}p < 0.01$ (post-hoc Sidak's comparisons vs. pre-phase; only controls) after significant effects in ANOVA.

post-phase [ts < 1.19, ps > 0.56]. Further comparisons revealed that locomotor activity during playback of lion urine-induced 22-kHz calls was significantly lower than in the pre- and post-phases [ts > 5.10, ps < 0.0001].

Very similar effects were observed during the playback of fear CS-induced 22-kHz calls (Fig. 5B). Time course analysis (left panel) showed that there was a tendency for an attenuation of locomotor activity during playback [ANOVA: interaction stimulus x time: $F_{10,190} = 1.70$, $p = 0.08$; time: $F_{10,190} = 4.82$, $p < 0.0001$; stimulus: $F_{1,19} = 0.25$, $p = 0.62$]. However, analysis of the phases revealed a playback effect [ANOVA: interaction stimulus x time: $F_{2,38} = 3.39$, $p = 0.04$; phase: $F_{2,38} = 18.64$, $p < 0.0001$; stimulus: $F_{1,19} = 1.31$, $p = 0.27$]. Post-hoc tests confirmed a significant difference in locomotor activity during the playback presentations of the fear CS-induced 22-kHz calls and the respective controls [$t = 3.27$, $p = 0.007$], as well as during playback of the fear CS-induced 22-kHz calls and the pre- and post-phases [ts > 5.07, ps < 0.0001].

Playback of both types of 22-kHz calls at a higher intensity (Fig. 5C,D) did not induce a specific locomotor activity response when compared with their respective controls [ANOVAs: interactions: Fs < 0.90, ps > 0.53; stimulus: Fs < 0.15, ps > 0.70]. However, there was a strong effect of time [Fs > 4.35, ps < 0.0001]. Post-hoc comparisons showed that locomotor activity was reduced during and after playback of all stimuli at higher intensity, i.e. lion urine induced-22-kHz calls, fear CS-induced 22-kHz calls, and their respective controls [comparison with pre-phase: ts > 2.78, ps < 0.02].

Discussion

The aim of our first experiment was to investigate defensive behaviours of rats during exposure to predator odours. We used two natural predator odours, samples of fox and lion urine respectively, as well as the synthetic predator odour TMT, a component of fox odor[38,39]. Notably, both natural and synthetic predator odours were able to induce overt defensive behaviours, such as avoidance behaviour. However, a clear dissociation regarding ultrasonic vocalizations was detected. Whereas samples of lion or fox urine induced 22-kHz calls in approximately one

fifth of the exposed rats, no single animal emitted 22-kHz calls upon exposure to TMT. In our second experiment, we exposed a different group of rats to playbacks of the 22-kHz calls recorded in the first experiment as well as to fear CS-induced 22-kHz calls. While high intensity playbacks led to unspecific effects, specific effects were seen under low intensity conditions, with 22-kHz calls inducing behavioural inhibition as reflected by a reduction in distance travelled in the radial maze.

Our data support the general finding that both natural and synthetic predator odours are able to induce overt defensive behaviours in rats that are naive to these odors[10,14,25,26]. In the present study, TMT and samples of lion urine induced avoidance behaviour measured by a reduction in contact time with the sample, as well as less time spent in the area in which the sample was presented. Additionally, TMT induced a significant increase in immobility, one of the most prominent behavioural effects of TMT[24,40]. Surprisingly, exposure to fox urine did not induce overt defensive behaviour in the present experiment, although this has been shown in previous studies of our group[20,41–44] and of others[45–47]. A potential reason for this may be that the experimental protocol of the present study differed from the one used before. However, since we detected robust effects of lion urine samples and TMT, we abstained from optimizing our protocol for fox urine. The robust effects of lion urine are very similar to those published before[23] and can be explained by the approximately 25 times higher concentration of 2-phenylethylamine in lion urine than in fox urine[23]. Previously, we demonstrated that 2-phenylethylamine is key component of predator urine triggering defensive behavior[23].

In the present study, exposure to both samples of predator urine induced 22-kHz calls. This is in accordance with the findings of Webber and colleagues[34] who also demonstrated that exposure to a predator odour is able to induce 22-kHz calls in rats. Whereas Webber and colleagues detected a mean of 8 ± 7 calls in a 5-min exposure session to a cat collar, the present study – if non-calling animals are included in the calculation – revealed a mean of about 18 ± 8 calls in a 10-min exposure session to lion urine. Although it is not explicitly stated in the Webber *et al*. publication, only approximately 30% of the rats in this study emitted 22-kHz calls (H.C. Cromwell, personal communication, September 25, 2017). The low proportion of vocalizing animals in the Webber *et al*. study and in the present study clearly indicate that predator odour does not seem to be a very reliable inducer of 22-kHz calls. This is much less compared with for example fear conditioning studies, in which usually more than half of the rats, sometimes almost all of them, emit 22 kHz calls[30,48]. Nevertheless, the number of emitted calls in the present experiment (70 calls/10 min; Fig. 3) is highly comparable to what has been observed in a fear conditioning experiment with moderate aversive stimuli[30]. In this context, it is important to note that the acoustic parameters of our recorded calls did not differ from 22-kHz calls recorded during fear conditioning[30], during handling[49], after air puffs[50], after acoustic startle stimuli[51], as well as during cat exposure[33], indicating the same nature of the calls.

An interesting observation of our study is that exposure to TMT did not induce 22-kHz calls, despite overt defensive behaviour was more pronounced with TMT and that the odour intensity of TMT was much higher than of the predator urine samples. This finding is remarkable in the face of the discussion whether TMT is really perceived by the animals as a predator odour or not[25,52–54]. If TMT has not the ability to induce 22-kHz calls, a well-established species-specific defensive behaviour of rats, this would argue for the idea that TMT does not represent a predator odour. In fact, a recent study raises severe doubts about whether TMT is a component of fox feces[38], since it was not reliably detectable[55]. Clearly, more studies are necessary to clarify the origin and the properties of TMT.

Our observation that predator odour induced 22-kHz calls leads to the obvious question why rats emit 22-kHz calls during exposure to predator odour or to the predator itself. Notably, the emission of 22-kHz calls is only one of many defensive behaviours in rats and usually not the first that is expressed after encountering a potentially dangerous situation[56–58]. Following the predatory imminence continuum, usually risk-assessment behaviours are first expressed (if the danger is not too immediate), followed by avoidance, escape behaviour (if there are possibilities to do so) or freezing behaviour (if there are no possibilities to hide, avoid, or escape). Ultrasonic vocalization is usually observed after the immediate and active defensive responses[32], often during freezing behavior[28,49], with latencies in the minute-range. A potential drawback of 22-kHz call emission might be that these calls are well audible to a substantial number of predators including cats, dogs, and foxes[59] and thereby guide the attention of the predators to the emitting animal. However, the 22-kHz calls are discussed to have a communicative function, i.e. to serve as alarm calls to warn conspecific about potential danger[28,29]. If they have this function, these calls should be able to affect the emotional state of receiver animals[29] and thereby also change behaviour of these.

The latter motivated us to perform our second experiment. In this experiment, animals were put on a radial maze and exposed to playback presentations of the lion urine-induced 22-kHz calls recorded in the first experiment. We previously demonstrated that playback presentations of 50-kHz calls but not 22-kHz calls induce approach behaviour, with rats spending more time on the arms of the radial maze next to the loudspeaker[36,37,60]. In the present study, we did not only present the lion urine-induced 22-kHz calls but also 22-kHz calls induced by an auditory fear CS from a fear conditioning experiment. As control stimuli we used phase-scrambled and frequency-shifted versions of the recordings mentioned before. Furthermore, we used two different intensities of the playbacks. The first one was in the 40–50 dB SPL range, the second one in the 70–80 dB SPL range. The high intensity represents the call intensity measured approximately 15 cm away from the call-emitting animal[49], whereas the low intensity is reached approximately 3–4 m away from a calling rat, a distance which might be normal in a rat pack foraging in nature. Under low intensity conditions, 22-kHz calls (induced by exposure to both lion urine and fear CS) induced a reduction of locomotor activity. This reduction was only observed (1) during but not after playback, and (2) only during playback presentations of the natural 22-kHz calls but not of their respective controls. Although no effects of 22-kHz calls were seen in some studies[61–65], an attenuation of locomotor activity during or after playback presentations of 22-kHz calls has already been described[36,66,67]. In these studies, noise stimuli, constant sine wave tones, or 50-kHz calls were used as controls. However, in the present study, phase-scrambled and frequency-shifted versions of the natural 22-kHz calls were used as controls. This approach might be more appropriate, particularly because these controls share more acoustic key features with natural

22-kHz calls, particularly total calling time and temporal patterning. The fact that the acoustic control stimuli – at low intensities – did not induce a behavioural response might indicate that rats recognize specific features of the natural calls and do not respond to other similar stimuli in the same way. The difference in behavioural responses between natural 22-kHz calls and their respective controls was most likely driven by the frequency shift. Controls were shifted up in frequency by 25 kHz, i.e. clearly out of the frequency range in which 22-kHz calls typically occur. The effects of phase-scrambling were comparatively mild. This is because 22-kHz calls are typically characterized by very low levels of frequency modulation. The most prominent effect of phase scrambling was that the typical downward slope of 22-kHz calls is completely removed. However, because very little is known about specific acoustic features involved in alarm communication through 22-kHz calls future studies appear warranted. For instance, it would be interesting to present 22-kHz calls originating from several individual rats to link differences in specific acoustic features of the 22-kHz calls between senders to the behavioural response patterns evoked in receivers. Moreover, selective experimental manipulations of individual acoustic features appear of interest. This would also help to rule out the possibility that the behavioural responses observed in the present study are associated with peculiarities in the 22-kHz calls applied here or to the playback treatment in general[68] - although the similarity of the response patterns evoked by natural lion urine-induced 22-kHz calls and fear CS-induced 22-kHz calls clearly speaks for a general effect, particularly when considering the prominent differences in acoustic features between the two stimuli. Notably, the behavioural response to playback presentations was also quite modest, i.e. an attenuation of locomotor activity without avoidance or flight reactions. However, such a response might be adaptive since it helps to identify the actual source of the threat.

With the higher intensity, the behavioural effects of the playback presentations became unspecific, i.e. there was also a response to the respective controls. Furthermore, an attenuation of locomotor activity was not only induced during but also after the presentation of the playback, very similar to the effects described by Brudzynski and Chiu[66]. This unspecific and more pronounced behavioural effect might be adaptive if a potential danger is very close[58], which might here be indicated by the loudness of the playback.

In summary, the present data demonstrate that rats express overt defensive behaviour and emit 22-kHz calls when exposed to samples of predator urine. TMT only induced overt behaviour but no 22-kHz calls. Playback of the recorded 22-kHz calls attenuated locomotor activity in another group of rats, indicating that these calls are recognized and transmit information.

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Author Contributions
M.F., K.W. and M.W. designed the study. M.B., K.W. and M.W. performed the experiments. M.F. and K.W. analysed the data and compiled the figures. M.F., K.W. and M.W. wrote the manuscript. All authors read, critically revised, and approved the final manuscript.

Additional Information
Competing Interests: M.W. is scientific advisor of Avisoft Bioacoustics. The other authors declare no competing interests.

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STUDIE III

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Reduced emission of alarm 22-kHz ultrasonic vocalizations during fear conditioning in rats lacking the serotonin transporter

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ABSTRACT

Rats display a rich social behavioral repertoire. An important component of this repertoire is the emission of whistle-like calls in the ultrasonic range, so-called ultrasonic vocalizations (USV). Long low-frequency 22-kHz USV occur in aversive situations, including aggressive interactions, predator exposure, and electric shocks during fear conditioning. They are believed to reflect a negative affective state akin to anxiety and fear. A prominent theory suggests that 22-kHz USV function as alarm calls to warn conspecifics. Serotonin (5-hydroxytryptamine, 5-HT) is strongly implicated in the regulation of affective states, particularly anxiety and fear. A key component of the system is the 5-HT transporter (5-HTT, also known as SERT), regulating 5-HT availability in the synaptic cleft. In the present experiment, we studied the effects of SERT deficiency on overt fear-related behavior and alarm 22-kHz USV during fear conditioning in male and female rats. While overt fear-related behavior was not affected by SERT deficiency and sex, the emission of alarm 22-kHz USV was clearly reduced in homozygous $SERT^{-/-}$ but not heterozygous $SERT^{+/-}$ mutants, as compared to their wildtype $SERT^{+/+}$ littermate controls. Genotype effects were particularly prominent in females. Females in general emitted fewer alarm 22-kHz USV than males. This supports the view that 22-kHz USV are, at least partly, independently regulated from anxiety or fear and as socially mediated alarm calls do not simply express a negative affective state. Reduced 22-kHz USV emission in rats lacking SERT might be due to social deficits in the use of 22-kHz USV as a socio-affective signal to warn conspecifics about threats.

1. Introduction

Rats display a rich social behavioral repertoire (Ellenbroek and Youn, 2016; Homberg et al., 2017). An important component of this repertoire is the emission of whistle-like calls in the ultrasonic range, so-called ultrasonic vocalizations (USV; Brudzynski, 2013; Knutson et al., 2002; Wöhr and Schwarting, 2013). Two main types are typically distinguished in juvenile and adult rats. Firstly, short high-frequency 50-kHz USV occur in appetitive situations, most notably social play (Knutson et al., 1998; Lukas and Wöhr, 2015) and mating (Barfield et al., 1979; Sales, 1972b). Such 50-kHz USV are believed to reflect a positive affective state akin to joy or happiness (Panksepp and Burgdorf, 2000). They appear to play an important role in regulating affiliative social interactions as contact calls (Wöhr, 2018) and elicit social approach behavior in receiver rats (Wöhr and Schwarting, 2007).

Secondly, long low-frequency 22-kHz USV occur in aversive situations. This includes aggressive interactions with conspecifics (Sales, 1972a; Vivian and Miczek, 1993) and exposure to predators or their odors (Blanchard et al., 1991; Fendt et al., 2018). In the laboratory, 22-kHz USV are typically evoked by administering air puffs (Brudzynski and Holland, 2005; Inagaki and Mori, 2015), acoustic startle stimuli (Kaltwasser, 1991; Vivian et al., 1994), and electric shocks (Cuomo et al., 1988; Tonoue et al., 1986), often serving as an additional measure in fear conditioning experiments (Molewijk et al., 1995; Rowan et al.,

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1990). They are believed to reflect a negative affective state akin to anxiety and fear (Brudzynski, 2019). In support of that view, it was shown that 22-kHz USV emission covaries with the aversiveness of a situation such that rats exposed to higher foot shock intensities vocalize more and louder during fear conditioning than rats exposed to less intense foot shocks (Wöhr et al., 2005). Potentiated 22-kHz USV emission is seen in rats characterized by higher trait anxiety (Borta et al., 2006) and following prenatal immune activation (Yee et al., 2012a), maternal neglect (Wöhr and Schwarting, 2008a), and juvenile stress exposure (Yee et al., 2012b). At the neurobiological level, amygdala and central grey were shown to be involved in regulating the emission of 22-kHz USV (Choi and Brown, 2003; Yajima et al., 1980).

A prominent theory suggests that 22-kHz USV function as alarm calls to warn conspecifics about threats. This view originates from a series of experiments on antipredator defensive behavior assayed in social groups of rats living in a visible burrow system (Blanchard et al., 1991; Blanchard et al., 1992). Rats exposed to a cat under such semi-natural conditions were found to retreat to the burrow, where they start to emit 22-kHz USV. Importantly, 22-kHz USV emission was reported to be potentiated by the presence of conspecifics and to evoke a fear response in receiver rats. The latter was also observed during social fear conditioning (Kim et al., 2010; Wöhr and Schwarting, 2008b) and confirmed in playback studies, where 22-kHz USV induced behavioral inhibition in receiver rats (Brudzynski and Chiu, 1995; Fendt et al., 2018).

In preclinical research, alarm 22-kHz USV emission was repeatedly used for testing the efficacy of novel potential anxiolytic compounds and antidepressant drugs, primarily targeting serotonin (5-hydroxytryptamine, 5-HT; Sánchez, 2003; Wöhr and van Gaalen, 2018). 5-HT is an important modulatory neurotransmitter in the mammalian brain. The 5-HT system is complex, including a large number of 5-HT receptors, together with enzymes involved in 5-HT synthesis and degradation (Descarries et al., 2010). A key component of the system is the 5-HT transporter (5-HTT, also known as SERT), regulating 5-HT availability in the synaptic cleft through reuptake of 5-HT into the presynaptic terminal (Murphy and Lesch, 2008).

The majority of pharmacological studies on alarm 22-kHz USV were targeting primarily 5-HT1 and to a lesser extent also 5-HT2 receptor families (Baudrie et al., 1993; de Vry et al., 1993; Molewijk et al., 1995; Schreiber and de Vry, 1993; Sánchez, 1993; Sommermeyer et al., 1993). Full or partial 5-HT1A receptor agonists, including 8-OH-DPAT, buspirone, gepirone, and ipsapirone, were consistently reported to block the emission of 22-kHz USV (Sánchez, 2003; Wöhr and van Gaalen, 2018). Another primary target of pharmacological studies was SERT and an extensive series of experiments showed that selective serotonin reuptake inhibitors (SSRIs) antagonize 22-kHz USV emission (Kassai and Gyertyán, 2018; Sánchez, 2003).

The polymorphic region in the promoter of the SERT gene (5-HTTLPR) leads to the formation of short and long allelic variants in humans, with the short variant resulting in reduced transcription and altered function of SERT (Canli and Lesch, 2007). The short allelic variant was associated with increased neuroticism, which includes higher anxiety levels (Lesch et al., 1996). This trait contributes to a greater risk of developing depressive symptoms following early life stress (Caspi et al., 2003) and is paralleled by anatomical and functional changes in several brain regions, such as higher levels of amygdala activation in response to fearful stimuli (Hariri et al., 2002).

To model altered 5-HT function, mouse and rat studies use the genetic modification of SERT availability by partially or fully reducing SERT expression (Holmes et al., 2003; Homberg et al., 2007a). In rats, the complete absence of SERT leads to a prominent increase in basal extracellular 5-HT levels and unresponsiveness to the SSRI citalopram (Homberg et al., 2007a). Similar to short allele carriers in humans, rats lacking SERT display elevated levels of anxiety-related behavior in unconditioned tasks, such as open field, elevated plus maze, and novelty suppressed feeding (Golebiowska et al., 2019; Olivier et al., 2008b; Schipper et al., 2011). Depression-related behavior is increased, as reflected in higher levels of immobility in the forced swim test and reduced sucrose consumption (Olivier et al., 2008b). Furthermore, social behavior is impaired, most notably social play in juveniles (Homberg et al., 2007c) and reciprocal social interactions in adulthood (Golebiowska et al., 2019; Schipper et al., 2011). Fear extinction is impaired (Luoni et al., 2013; Nonkes et al., 2012; Schipper et al., 2011; Schipper et al., 2018; Schipper et al., 2019a; Schipper et al., 2019b; Shan et al., 2014; Shan et al., 2018). Consistent phenotypes were obtained in mice (Kalueff et al., 2010).

Despite the fact that 5-HT is strongly implicated in the regulation of 22-kHz USV emission and the important role SERT plays in anxiety and fear, it is not yet known how SERT availability affects the emission of 22-kHz USV during fear conditioning in rats. In the present experiment, we studied the effects of SERT deficiency on overt fear-related behavior and alarm 22-kHz USV during fear conditioning in rats, by comparing male and female constitutive homozygous $SERT^{-/-}$ and heterozygous $SERT^{+/-}$ mutants to their wildtype $SERT^{+/+}$ littermate controls. The affective state hypothesis suggests that rats lacking SERT emit more 22-kHz USV because 22-kHz USV reflect a negative affective state akin to anxiety and fear and SERT deficiency leads to increased levels of anxiety-related behavior and impaired fear extinction. Alternatively, the communicative function hypothesis predicts that rats lacking SERT emit fewer 22-kHz USV because 22-kHz USV function as socially mediated alarm calls and SERT deficiency leads to social deficits.

2. Materials and methods

2.1. Animals and housing

Effects of SERT deficiency on overt fear-related behavior and alarm 22-kHz USV were tested in male and female constitutive homozygous $SERT^{-/-}$ and heterozygous $SERT^{+/-}$ mutant rats, as compared to their wildtype $SERT^{+/+}$ littermate controls. $SERT^{-/-}$ rats completely lacking 5-HTT (SLC6A41Hubr) were generated by N-ethyl-N-nitrosurea (ENU) induced mutagenesis (Smits et al., 2006) and outcrossed with commercially available Wistar rats (Harlan, Ter Horst, the Netherlands) for at least 10 generations (Homberg et al., 2007a). In total, $N = 81$ rats were used, including $N = 47$ female rats ($SERT^{+/+}$: $N = 15$, $SERT^{+/-}$: $N = 21$, $SERT^{-/-}$: $N = 11$) and $N = 34$ male rats ($SERT^{+/+}$: $N = 10$, $SERT^{+/-}$: $N = 15$, $SERT^{-/-}$: $N = 9$).

A heterozygous breeding strategy was applied to obtain $SERT^{-/-}$ and $SERT^{+/-}$ offspring together with $SERT^{+/+}$ littermate controls. Briefly, $SERT^{+/-}$ males and females were paired for breeding. To avoid genetic drifts, male and female $SERT^{+/-}$ breeders were obtained by outcrossing $SERT^{+/-}$ males with Wistar females (Harlan, Ter Horst, the Netherlands). Approximately 2 weeks after pairing for breeding, females were individually housed and inspected daily for pregnancy and delivery. The day of birth was defined as postnatal day (PND) 0. In order to avoid litter effects, only litters with all three genotypes were included in the experiments. Breeding was performed at the Faculty of Psychology, Philipps-University of Marburg, Germany.

After weaning on PND 21, rats were socially housed in mixed-genotype groups of 4–6 with same-sex littermate partners in standard Macrolon Type IV cages with bedding and high stainless steel covers ($58 \times 33 \times 20$ cm) under standard laboratory conditions. Cages were kept in temperature- and humidity-controlled rooms ($22 \pm 2\,^{\circ}C$, 30–60%) with a 12:12 h light/dark cycle (lights on at 7 am). Standard rodent chow (Altromin, Lage, Germany) and water (0.0004% HCl solution) were available *ad libitum*. Rats were repeatedly handled and had been used in other behavioral paradigms before, namely, in the following order, pup ultrasonic calling, playback of 50-kHz USV, open field, elevated plus maze, and amphetamine-induced 50-kHz USV (once 2.5 mg/kg amphetamine). Approximately 3–4 weeks later, fear conditioning took place at the age of ~4 months (females weighing between 190 and 261 g and males weighing between 243 and 455 g). Rats were handled prior to fear conditioning in a standardized way for 5 min.

Behavioral testing was performed by an experimenter blind to the rat's genotype and conducted during the light cycle between 7 am and 7 pm.

All procedures were conducted in 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 (Tierschutzbehörde, Regierungspräsidium Gießen, Germany).

2.2. Genotyping

Rat tail snips were collected by dissecting ~0.3 cm of tail on PND 5. Tails were digested, genomic DNA was isolated and purified using the Qiagen DNAeasy Blood & Tissue Kit according to the manufacturer's instructions (Hilden, Germany). Genotyping was performed using KASP technology for allelic discrimination (LGC, Berlin, Germany) and results were analyzed with a LightCycler 480 (LC480, Roche Life Science, Mannheim, Germany) by c.ATG (Institut für Medizinische Genetik und Angewandte Genetik, Universitätsklinikum Tübigen, Germany). 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.

2.3. Fear conditioning

Fear conditioning was performed as previously described (Borta et al., 2006). Briefly, testing took place in a shock chamber (335 × 350 × 380 mm) made of grey and transparent plastic walls. The roof and one wall were made of transparent plastic to allow video observation. A loudspeaker was mounted in one wall ~30 cm above the floor for presenting tones. The floor of the shock chamber was made of stainless steel rods (diameter: 5 mm) spaced 1 cm apart. An UltraSoundGate Condenser CM 16 Microphone (Avisoft Bioacoustics, Berlin, Germany) was attached to the roof of the shock chamber ~30 cm above the floor. The chamber was housed in a sound attenuating isolation cubicle (510 × 710 × 510 mm; Coulbourn Instruments, Allentown, USA) and equipped with two white light LED spots (~40 lx, Conrad Electronic, Hirschau, Germany) and a black and white CCD camera (Conrad Electronic).

As the conditioned stimulus (CS), a 3-kHz sinewave tone (generated with: GoldWave Digital Audio Editor) was presented at 72 dB for 20 s. As the unconditioned stimulus (UCS), a 0.5 mA scrambled shock (52 Hz, 120 V peak-to-peak amplitude) was applied via the rod floor using a stand-alone shocker (Med Associates, St. Albans, USA) during the last 500 ms of the tone. A shock intensity of 0.5 mA was previously identified as the lowest shock intensity needed for inducing unconditioned and conditioned 22-kHz USV in a reliable manner (Wöhr et al., 2005), while still allowing the detection of inter-individual differences in 22-kHz USV emission (Borta et al., 2006). Stimulus delivery and timing were controlled by the Presentation program (Neurobehavioral Systems, Albany, USA).

Testing was performed on 3 consecutive days. On the first day (termed habituation), each rat was placed in the shock chamber for 11 min without CS or UCS presentation to habituate it to the test environment. On the second day (termed acquisition), i.e. after 24 h, each animal was placed again into the shock chamber for 11 min. After an initial phase of 3 min where no tone or shock was given, the rat was exposed to six CS/UCS pairings, each followed by an inter-stimulus interval (ISI) of 60 s, during the subsequent conditioning phase. On the third day (termed extinction), the rat was again placed into the shock chamber for 11 min. After an initial context phase of 3 min, the CS was presented 6 times for 20 s, each followed by an ISI of 60 s, during the subsequent cue phase. Prior to each rat, the equipment was cleaned with 0.1% acetic acid solution.

2.4. Analysis of ultrasonic vocalizations

The UltraSoundGate Condenser CM 16 microphone (Avisoft Bioacoustics) used for USV recordings was connected via an UltraSoundGate 416 USB audio device (Avisoft Bioacoustics) to a 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 (Avisoft Bioacoustics). The microphone is sensitive to frequencies of 15–180 kHz with a flat frequency response (±6 dB) between 25 and 140 kHz.

For acoustical analysis, recordings were transferred to Avisoft SASLab Pro (Version 4.2, Avisoft Bioacoustics). 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). A lower-cut-off-frequency of 18 kHz was used to reduce background noise outside the relevant frequency band to 0 dB. Detection of 22-kHz USV was provided by an automatic threshold-based algorithm (threshold: −40 dB) and a hold time mechanism (hold time: 20 ms). An experienced user checked the accuracy of 22-kHz USV detection and obtained a 100% concordance between automatic and observational detection. Total number of 22-kHz USV, total calling time, and the latency to emit the first 22-kHz USV were determined. Dependent on the time point of their occurrence, 22-kHz USV were divided into those emitted during CS presentations or those emitted within ISIs. Moreover, a detailed spectrographic analysis was performed and various acoustic features were analyzed. Specifically, for each 22-kHz USV, call duration, peak frequency, peak amplitude, and frequency modulation were determined. Peak frequency and peak amplitude were automatically 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.

As 22-kHz USV are emitted either as single pulses or in short bouts, calls were divided into those starting a bout versus those within a bout according to the duration of the interval between two calls, i.e. the inter-call interval. Based on detailed studies on the bout structure of 22-kHz USV emission (van der Poel et al., 1989; van der Poel and Miczek, 1991), a bout was defined as a call, or a number of calls, separated from other calls by inter-call intervals longer than 320 ms, i.e. the inter-bout interval. To describe the temporal patterning of 22-kHz USV production, the number of bouts and the number of calls within a bout, i.e. bout length, were determined, as temporal patterning was previously shown to be dependent on the aversiveness of the situation (van der Poel and Miczek, 1991), to be affected by early life stress (Yee et al., 2012a), and to be modulated by anxiolytics (van der Poel et al., 1989).

In addition to the typical 22-kHz USV, low-short 22-kHz USV were counted (Brudzynski et al., 1993). Low-short 22-kHz USV were defined as calls with a peak frequency below 32 kHz and a call duration shorter than 300 ms, but their behavioral significance has not yet been determined (Brudzynski, 2013; Brudzynski, 2019). Finally, 50-kHz USV were counted.

2.5. Analysis of overt behavior

Overt behavior was scored from video recordings by an experienced observer blind to the rat's genotype using The Observer XT (Noldus, Wagenigen, the Netherlands). The following behavioral measures were scored: duration of immobility, duration of grooming, and number of rearings. Immobility was defined as the suppression of all motor activity, but respiration-related motions. Grooming included face, body, and genital grooming movements. Rearing was defined as standing on hind legs with front legs not being in contact with the floor. Overt behaviors were scored in 20 s time bins, including CS phases, i.e. before CS presentation (preCS), during CS presentation (CS), and after CS presentation (postCS), together termed trial. For the acquisition day, detailed temporal analyses linking overt behavior and 22-kHz USV were

performed by means of high-resolution ethograms using The Observer XT, including time phases in which rats displayed immobility, grooming, rearing, and other motor activity. The term other motor activity was used for time phases where no immobility, grooming, or rearing was displayed.

2.6. Hot plate

After fear conditioning, a hot plate test was performed under red light (~300 lx) to assess the effects of SERT deficiency on pain reactivity to thermal stimulation (precision hot plate, Prezitherm PZ35; Harry Geistigkeit GmbH, Düsseldorf, Germany). On the first day, the rat was placed onto the unheated apparatus for 120 s to habituate it to the test environment. On the next day, the rat was placed into the center of the hot plate kept at a constant temperature of 52 °C. The time to lick one of the four paws was measured by observation. To prevent tissue damage, a cut-off latency of 30 s was applied.

2.7. Statistical analysis

All statistical tests were carried out using IBM SPSS Statistics (Version 24.0) software. To compare body weight and pain sensitivity as well as immobility, grooming, and rearing displayed during acquisition and extinction, two-way ANOVAs with the between-subject factors genotype (G) and sex (S) were calculated. Separate two-way ANOVAs were calculated for the initial phase of acquisition, the subsequent conditioning phase, the initial context phase of extinction, and the cue phase of extinction. Repeated measures three-way ANOVA with the between-subject factors G and S and the within-subject factor time bin (T) were conducted to test whether the time courses were affected. To compare immobility, grooming, and rearing displayed during CS presentations, repeated measures three-way ANOVA with the between-subject factors G and S and the within-subject factor CS presentation (CS) were performed. When focusing the analysis on ISIs, repeated-measures ANOVAs with the between-subject factors G and S and the within-subject factors ISI and T were calculated. For the analysis of trials, repeated-measures ANOVAs with the between-subject factors G and S and the within-subject factors CS phase (CSP) and trial (TRL) were calculated.

To compare the number of rats emitting 22-kHz and 50-kHz USV between genotypes and sexes, chi^2-tests were performed. For comparing the emission of 22-kHz and 50-kHz USV, including acoustic features, two-way ANOVAs with the between-subject factors G and S were calculated. ANOVAs were followed by post-hoc LSD tests when appropriate. Paired *t*-tests were applied to compare 22-kHz USV emission between CS and ISI or between different overt behaviors. To correlate 22-kHz USV emission and overt behavior, Pearson correlation coefficients were calculated. For graphical representations of acoustic parameters as percentages, only rats emitting more than five 22-kHz USV per individual were included.

Since sphericity was not met for several repeated-measures ANOVAs, Greenhouse-Geisser corrected values are reported. To avoid alpha error accumulation, the two-tailed significance threshold was set to 1% throughout the confirmatory testing focused on overt fear-related behavior. For the exploratory analysis of 22-kHz and 50-kHz USV, the two-tailed significance threshold was set to 5%. All values are reported as mean ± standard error of the mean (SEM).

3. Results

3.1. Overt fear-related behavior – acquisition

While immobility was low during the first day of habituation (data not shown), rats displayed high levels of immobility following repeated tone-shock pairings during acquisition on the second day. Time spent immobile during acquisition, however, did not differ between genotypes and sexes, with no evidence for genotype x sex interactions (G: $F_{2,75} = 0.686$, $p = .507$; S: $F_{1,75} = 0.693$, $p = .408$; GxS: $F_{2,75} = 0.939$, $p = .395$). Specifically, during min 1–3, the initial phase of acquisition, where no tone-shock pairings were applied yet, immobility was absent irrespective of genotype and sex (G: $F_{2,75} = 0.610$ $p = .546$; S: $F_{1,75} = 0.033$, $p = .856$; GxS: $F_{2,75} = 1.990$, $p = .144$; Fig. 1A, A‘). During the subsequent conditioning phase, min 4–11 of acquisition with repeated tone-shock pairings, a prominent increase in immobility was evident, but the immobility response was not affected by genotype and sex (G: $F_{2,75} = 0.640$ $p = .530$; S: $F_{1,75} = 0.674$, $p = .414$; GxS: $F_{2,75} = 0.865$, $p = .425$; Fig. 1B, B‘). In fact, the increase in immobility occurred irrespective of genotype and sex (T: $F_{11.432,857.427} = 135.852$, $p < .001$; all *p*-values for interactions with $T > 0.010$; Table 1; Fig. 1C, C‘).

When analyzing CS presentations separately, immobility increased with repeated CS presentations, with a prominent increase from the first to the second CS presentation (CS: $F_{4.355,326.604} = 33.840$, $p < .001$). Genotype and sex, however, had no effect on immobility during CS presentations (G: $F_{2,75} = 0.495$, $p = .611$; S: $F_{2,75} = 0.621$, $p = .433$; GxS: $F_{2,75} = 2.066$, $p = .134$; all *p*-values for interactions with CS >0.010; Table 1).

When focusing the analysis on ISIs, each including three 20s time bins, there was a gradual increase in immobility with repeated ISIs (ISI: $F_{4.395,329.654} = 9.839$, $p < .001$). This increase occurred irrespective of genotype (G: $F_{2,75} = 0.739$, $p = .481$; ISIxG: $F_{8.791,\ 329.654} = 1.392$, $p = .192$), but was modulated by sex (S: $F_{2,75} = 0.635$, $p = .428$; GxS: $F_{2,75} = 0.500$, $p = .608$; ISIxS: $F_{4.373,329.654} = 3.648$, $p = .005$; ISIxGxS: $F_{8.791,329.654} = 1.225$, $p = .279$), with males showing a more prominent increase than females. Moreover, immobility increased across the three 20s time bins within ISIs (T: $F_{1.773,132.964} = 536.121$, $p < .001$). Genotype and sex had no effect on this increase within ISIs (all *p*-values for interactions with T and ISIxT >0.010; Table 1).

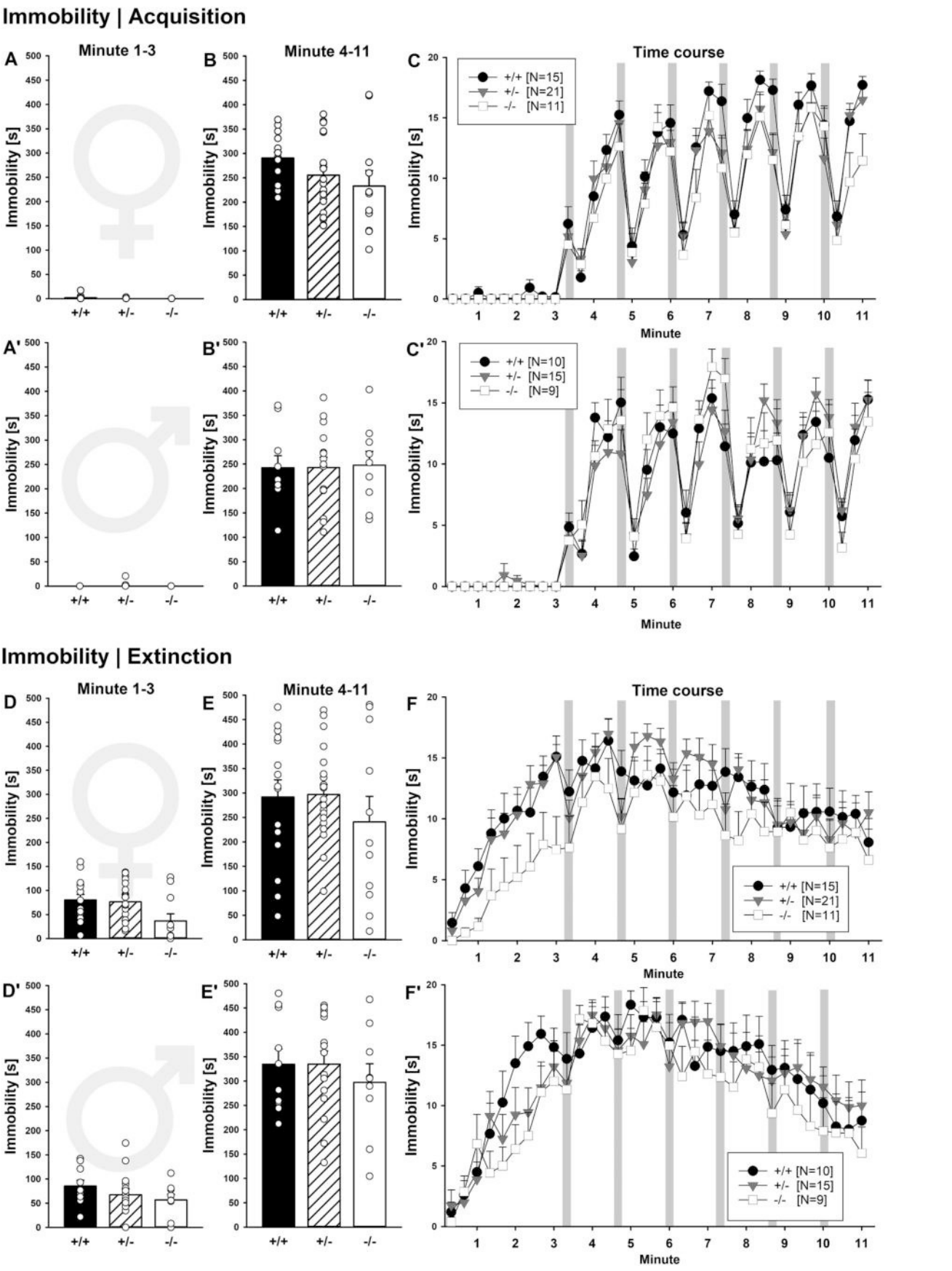
When focusing the analysis on trials, each including the three CS phases, i.e. preCS, CS, and postCS, immobility increased rapidly from the first to the second trial, with further moderate increases in subsequent trials (TRL: $F_{3.891,291.832} = 77.761$, $p < .001$). Within trials, immobility was highest during preCS and CS, but clearly lower during postCS immediately after the tone-shock pairings, partly because of escape-like responses to the shock (CSP: $F_{1.829,137.206} = 365.065$, $p < .001$). This temporal pattern emerged after the first trial and remained stable in subsequent trials (TRLxCSP: $F_{8.434,632.570} = 32.906$, $p < .001$). Neither genotype nor sex influenced this pattern (all *p*-values for interactions with TRL, CSP, and TRLxCSP >0.010; Table 1).

In summary, immobility during acquisition was not affected by genotype and sex. Likewise, no prominent effects of genotype and sex were found on rearing and grooming behavior (Table 1, Fig. 2 & Fig. 3).

3.2. Overt fear-related behavior – extinction

During extinction on the third day, time spent immobile did not differ between genotypes and sexes, with no evidence for genotype x sex interactions (G: $F_{2,75} = 1.457$, $p = .240$; S: $F_{1,75} = 3.022$, $p = .086$; GxS: $F_{2,75} = 0.376$, $p = .688$). Specifically, during min 1–3, the context phase of extinction, where no CS was presented yet, immobility did not differ between genotypes, independent of sex (G: $F_{2,75} = 4.145$, $p = .020$; S: $F_{1,75} = 0.299$, $p = .586$; GxS: $F_{2,75} = 0.761$, $p = .471$; Fig. 1D, D‘). Likewise, during the subsequent CS presentations in min 4–11, no effects of genotype and sex were evident (G: $F_{2,75} = 1.076$, $p = .346$; S: $F_{1,75} = 2.693$, $p = .105$; GxS: $F_{2,75} = 0.039$, $p = .962$; Fig. 1E, E‘). Irrespective of genotype and sex, immobility gradually increased during the context phase, peaked around min 3–5 during the cue phase, and slowly decreased towards the end of extinction (T: $F_{10.837,812.738} = 30.969$, $p < .001$; all *p*-values for interactions with $T > 0.010$; Table 1; Fig. 1F, F‘).

When analyzing CS presentations separately, an initial increase in immobility was evident, followed by a successive reduction with repeated CS presentations (CS: $F_{3.976,298.184} = 7.861$, $p < .001$).



(caption on next page)

Fig. 1. | Immobility.
Effects of SERT deficiency on the time spent immobile during acquisition (A-C') and extinction (D-F'). Depicted is the total time spent immobile during the first three minutes of acquisition and extinction, respectively, for female (A, D) and male (A', D') $SERT^{+/+}$ (black bar), $SERT^{+/-}$ (striped bar), and $SERT^{-/-}$ (white bar) rats, followed by the total time spent immobile during the CS presentation period in minute 4–11 (B, B'; E, E') and the detailed time course of immobility in successive 20s time bins during acquisition (C, C') and extinction (F, F') for $SERT^{+/+}$ (black circle), $SERT^{+/-}$ (grey triangle), and $SERT^{-/-}$ (white square) rats. Grey bars represent the 20s time bins during CS presentation. During acquisition, CS presentation was followed by a mild electric shock. $N = 47$ female rats (15 +/+, 21 +/−, 11 −/−), $N = 34$ male rats (10 +/+, 15 +/−, 9 −/−). Data presented as mean ± SEM.

Genotype and sex, however, had no effect on immobility during CS presentations (G: $F_{2,75} = 1.129$, $p = .329$; S: $F_{2,75} = 3.333$, $p = .072$; GxS: $F_{2,75} = 0.079$, $p = .924$; all p-values for interactions with CS >0.010; Table 1).

When focusing the analysis on ISIs, the pattern remains, yielding differences throughout the six ISIs (ISI: $F_{3.862,289.699} = 23.453$, $p < .001$), independent of genotype and sex (G: $F_{2,75} = 1.161$, $p = .319$; S: $F_{1,75} = 2.267$, $p = .136$; GxS: $F_{2,75} = 0.061$, $p = .941$; all p-values for interactions with ISI >0.010; Table 1). However, in contrast to acquisition, immobility did not increase across the three 20s time bins within ISIs (T: $F_{1.905,142.883} = 0.133$, $p = .867$). Genotype and sex had no effect (all p-values for interactions with T and ISIxT >0.010; Table 1).

When focusing the analysis on trials, immobility decreased with repeated trials (TRL: $F_{3.636,272.694} = 17.824$, $p < .001$). Within trials, the immobility response was particularly prominent immediately before and after CS presentation (CSP: $F_{1.798,134.851} = 15.834$, $p < .001$). This pattern was stable across trials (TRLxCSP: $F_{7.565,567.394} = 2.138$, $p = .034$) and was not affected by genotype and sex (all p-values for interactions with TRL, CSP, and TRLxCSP >0.010; Table 1).

In summary, immobility during extinction was not affected by genotype and sex. Likewise, no prominent effects of genotype and sex were found on rearing and grooming behavior (Table 1; Fig. 2 & Fig. 3).

3.3. Alarm 22-kHz USV – acquisition

During acquisition on the second day, the emission of 22-kHz USV was affected by genotype ($chi^2_2 = 9.400$, $p = .009$). While 60% of $SERT^{+/+}$ rats vocalized ($N = 15$ out of $N = 25$), only 15% of $SERT^{-/-}$ rats emitted 22-kHz USV ($N = 3$ out of $N = 20$). $SERT^{+/-}$ rats displayed an intermediate phenotype and 22-kHz USV occurred in 44% of cases ($N = 16$ out of $N = 36$). Moreover, the tendency to call was influenced by sex ($chi^2_1 = 9.422$; $p = .002$), with a higher propensity in male than female rats. While 62% of males vocalized ($N = 21$ out of $N = 34$), only 28% of females emitted 22-kHz USV ($N = 13$ out of $N = 47$).

Consistently, the number of 22-kHz USV emitted was also affected by genotype (G: $F_{2,75} = 3.215$, $p = .046$; Fig. 4A), with $SERT^{-/-}$ rats emitting fewer calls than $SERT^{+/-}$ and $SERT^{+/+}$ littermate controls ($p = .037$ and $p = .035$, respectively). Moreover, females emitted fewer 22-kHz USV than their male conspecifics (S: $F_{1,75} = 7.781$, $p = .007$; GxS: $F_{2,75} = 2.393$, $p = .098$). When restricting the analysis to rats emitting 22-kHz USV, i.e. excluding non-vocalizing rats, the number of 22-kHz USV did not differ between genotypes and sexes (G: $F_{2,28} = 0.301$, $p = .742$; S: $F_{1,28} = 2.816$, $p = .104$; GxS: $F_{2,28} = 2.717$, $p = .083$). Reduced emission of 22-kHz USV in $SERT^{-/-}$ rats was, at least partly, driven by an increase in the latency to emit 22-kHz USV (G: $F_{2,75} = 6.446$, $p = .003$; Fig. 4B). It took $SERT^{-/-}$ rats longer to emit the first 22-kHz USV than $SERT^{+/-}$ and $SERT^{+/+}$ littermate controls ($p = .027$ and $p < .001$, respectively). Sex did not affect the latency to emit 22-kHz USV (S: $F_{1,75} = 2.232$, $p = .139$; GxS: $F_{2,75} = 2.036$, $p = .138$). The number of 22-kHz USV bouts was not affected by genotype and sex (G: $F_{2,28} = 1.132$, $p = .337$; S: $F_{1,28} = 0.123$, $p = .729$; GxS: $F_{2,28} = 2.042$, $p = .149$; Fig. 4C).

Besides the tendency to call, genotype had an effect on total calling time (G: $F_{2,75} = 3.699$, $p = .029$; Fig. 5A, A', 5A"). $SERT^{-/-}$ rats spent the least amount of time emitting 22-kHz USV, differing from $SERT^{+/-}$ and $SERT^{+/+}$ littermate controls ($p = .029$ and $p = .019$, respectively). Moreover, females spent less time calling than their male conspecifics (S: $F_{1,75} = 6.699$, $p = .012$, GxS: $F_{2,75} = 1.935$, $p = .152$). Notably, most prominent genotype and sex effects on total calling time were evident during ISIs (G: $F_{2,75} = 3.461$, $p = .036$; S: $F_{1,75} = 5.630$, $p = .020$; GxS: $F_{2,75} = 1.461$, $p = .239$), while no differences during CS presentations were detected (G: $F_{2,75} = 1.803$, $p = .172$; S: $F_{1,75} = 3.513$, $p = .065$; GxS: $F_{2,75} = 0.619$, $p = .541$). $SERT^{-/-}$ rats spent less time emitting 22-kHz USV during ISIs than $SERT^{+/-}$ and $SERT^{+/+}$ littermate controls ($p = .044$ and $p = .016$, respectively). This pattern was driven by a genotype- and sex-dependent change in calling behavior. While overall the average time spent calling per time bin was higher during ISIs than CS presentations ($T_{80} = 5.323$; $p < .001$), the tendency to vocalize more during ISIs, i.e. in the absence of CS presentations, was not seen in all experimental groups. In fact, this effect was not seen in male and female $SERT^{-/-}$ rats ($T_8 = 1.511$; $p = .169$ and $T_{10} = 1.000$; $p = .341$; respectively). In male and female $SERT^{+/+}$ rats, in contrast, 22-kHz USV emission clearly increased during ISIs ($T_9 = 2.978$; $p = .015$ and $T_{13} = 2.304$; $p = .037$; respectively). $SERT^{+/-}$ rats, however, displayed an intermediate phenotype. While the increase during ISIs was evident in $SERT^{+/-}$ males ($T_{14} = 3.899$; $p = .002$), such an increase was not seen in $SERT^{+/-}$ females ($T_{20} = 1.782$; $p = .090$).

Interestingly, the temporal organization of 22-kHz USV emission was primarily dependent on sex. Although mean call duration was influenced by genotype, genotype differences did not reach statistical significance in *post-hoc* tests (G: $F_{2,28} = 6.764$, $p = .004$; Fig. 5B, B', 5B"). However, prominent sex effects were evident and modulated by genotype (S: $F_{1,28} = 36.158$, $p < .001$; GxS: $F_{2,28} = 12.333$, $p < .001$), with females typically emitting longer 22-kHz USV than males^{--}. Moreover, while the number of 22-kHz USV bouts was similar in males and females, bout length was strongly affected by sex (S: $F_{1,28} = 13.940$, $p = .001$; Fig. 5C, C', 5C"), with bouts emitted by females containing less 22-kHz USV than bouts emitted by males. Genotype had no effect on bout length (G: $F_{2,28} = 1.014$, $p = .376$; GxS: $F_{2,28} = 1.298$, $p = .289$).

Similarly, sex but not genotype affected the acoustic characteristics of the 22-kHz USV. While mean peak frequency of 22-kHz USV did not differ between genotypes and sexes (G: $F_{2,28} = 0.316$, $p = .731$; S: $F_{1,28} = 2.951$, $p = .097$; GxS: $F_{2,28} = 0.151$, $p = .860$; Fig. 6A, A', 6A"), frequency modulation differed between sexes and this sex effect was modulated by genotype (G: $F_{2,28} = 1.986$, $p = .156$; S: $F_{1,28} = 30.564$, $p < .001$; GxS: $F_{2,28} = 8.056$, $p = .002$; Fig. 6B, B', 6B"). In general, 22-kHz USV emitted by females were characterized by higher levels of frequency modulation than the ones emitted by males. The sex difference was particularly prominent in $SERT^{-/-}$ rats. Peak amplitude of 22-kHz USV did not differ between genotypes but sexes, with males emitting louder 22-kHz USV than females (G: $F_{2,28} = 0.345$, $p = .711$; S: $F_{1,28} = 12.911$, $p = .001$; GxS: $F_{2,28} = 0.444$, $p = .646$, Fig. 6C, C', 6C").

By means of temporal analyses using high-resolution ethograms, the time phases in which rats displayed immobility, grooming, rearing, and other motor activity were differentiated and the time-matched occurrence of 22-kHz USV was determined. It was found that rats emitted 22-kHz USV primarily during phases of immobility. Substantially lower levels of 22-kHz USV were found during rearing and there were no 22-kHz USV during grooming (immobility vs. rearing: $T_{33} = 11.886$; $p < .001$). During time phases where no immobility, grooming, or rearing but other motor activity was displayed, fewer 22-kHz USV occurred than during immobility (immobility vs. other motor activity: $T_{33} = 1.818$; $p = .078$), but more than during rearing (other motor activity vs. rearing $T_{33} = 8.702$; $p < .001$). Specifically, rats emitted 57% of all 22-kHz USV during phases of immobility, whereas 41% of all 22-kHz USV were emitted during other motor activity, and only 2% during

Table 1
Results ANOVAs - immobility, rearing, grooming by genotype and sex during acquisition and extinction. Total – ANOVA total duration/number. Minute 1–3 – total duration/number during the first three minutes of acquisition/extinction. Minute 4–11 – total duration/number during CS presentation period. Time bin – ANOVA for repeated measurements for 33 time bins. CS - ANOVA for repeated measurements for 6 CS presentations. ISI – two-way ANOVA for repeated measurements for 6 ISI × 3 time bins each. Trial/CS phase - two-way ANOVA for repeated measurements for 6 trials consisting of 3 different CS phases (preCS, CS, postCS). df – degrees of freedom. F – F-value. p – p-value. Significant results ($p < .01$) are highlighted in *bold and italic*.

			Immobility - acquisition			Immobility - extinction			Rearing - acquisition			Rearing - extinction			Grooming - acquisition			Grooming - extinction		
		df error	**df**	**F**	***p***	**df**	**F**	***p***	**df**	**F**	***p***	**df**	**F**	***p***	**df**	**F**	***p***	**df**	**F**	***p***
Total	Genotype	75	2	0.686	0.507	2	1.457	0.240	2	0.951	0.391	2	3.647	0.031	2	0.184	0.832	2	1.272	0.286
	Sex	75	1	0.693	0.408	1	3.022	0.086	1	0.268	0.606	1	0.431	0.514	1	1.188	0.279	1	0.818	0.369
	Genotype x Sex	75	2	0.939	0.395	2	0.376	0.688	2	3.143	0.049	2	1.304	0.278	2	1.552	0.219	2	0.752	0.475
Minute 1–3	Genotype	75	2	0.610	0.546	2	4.145	0.020	2	0.089	0.915	2	4.450	0.015	2	0.143	0.867	***2***	***5.663***	***0.005***
	Sex	75	1	0.033	0.856	1	0.299	0.586	1	0.218	0.642	1	0.007	0.933	1	1.292	0.259	1	0.055	0.816
	Genotype x Sex	75	2	1.990	0.144	2	0.761	0.471	2	4.737	0.012	2	1.699	0.190	2	1.865	0.162	2	0.091	0.913
Minute 4–11	Genotype	75	2	0.640	0.530	2	1.076	0.346	2	1.844	0.165	2	0.853	0.430	2	0.749	0.476	2	0.502	0.607
	Sex	75	1	0.674	0.414	1	2.693	0.105	1	0.002	0.963	1	0.958	0.331	1	0.427	0.515	1	0.842	0.362
	Genotype x Sex	75	2	0.865	0.425	2	0.039	0.962	2	1.009	0.370	2	0.182	0.834	2	0.074	0.929	2	0.851	0.431
Time bin	Time bin	857.427	***11.432***	***135.852***	***0.000***	***10.837***	***30.969***	***0.000***	***15.009***	***35.094***	***0.000***	***9.973***	***10.080***	***0.000***	***6.046***	***7.969***	***0.000***	***5.844***	***5.973***	***0.000***
	Time bin x Genotype	857.427	22.865	0.935	0.551	21.673	0.891	0.606	30.018	0.927	0.580	19.947	1.208	0.240	12.091	0.962	0.451	11.688	0.792	0.655
	Time bin x Sex	857.427	11.432	1.744	0.057	10.837	0.782	0.656	15.009	0.757	0.727	9.973	1.392	0.180	6.046	0.508	0.910	5.844	1.113	0.354
	Time bin x Genotype x Sex	857.427	22.865	1.331	0.138	21.673	0.777	0.755	***30.018***	***1.998***	***0.001***	19.947	1.074	0.372	12.091	1.170	0.302	11.688	1.251	0.247
CS	Genotype	75	2	0.495	0.611	2	1.129	0.329	2	0.800	0.453	2	1.150	0.322	2	0.711	0.495	2	0.834	0.439
	Sex	75	1	0.621	0.433	1	3.333	0.072	1	0.120	0.730	1	1.753	0.190	1	0.026	0.873	1	0.139	0.710
	Genotype x Sex	75	2	2.066	0.134	2	0.079	0.924	2	1.277	0.285	2	0.396	0.675	2	1.098	0.339	2	0.928	0.400
	CS	326.604	***4.355***	***33.840***	***0.000***	***3.976***	***7.861***	***0.000***	***3.887***	***6.072***	***0.000***	4.101	0.127	0.975	1.014	1.970	0.164	1.895	2.351	0.102
	CS x Genotype	326.604	8.709	0.486	0.879	7.952	0.404	0.917	7.774	0.787	0.611	8.201	0.976	0.456	2.029	0.879	0.421	3.790	1.769	0.142
	CS x Sex	326.604	4.345	0.595	0.681	3.976	0.617	0.650	3.887	1.230	0.298	4.101	1.860	0.116	1.014	0.090	0.768	1.895	1.258	0.286
	CS x Genotype x Sex	326.604	8.709	1.921	0.050	7.952	1.065	0.388	7.774	1.903	0.061	8.201	0.901	0.517	2.029	1.047	0.357	3.790	0.475	0.744
ISI x Time bin	Genotype	75	2	0.739	0.481	2	1.161	0.319	2	2.008	0.141	2	0.662	0.519	2	0.362	0.698	2	0.406	0.668
	Sex	75	1	0.635	0.428	1	2.267	0.136	1	0.001	0.975	1	0.610	0.437	1	0.571	0.452	1	0.824	0.367
	Genotype x Sex	75	2	0.500	0.608	2	0.061	0.941	2	0.944	0.394	2	0.108	0.898	2	0.362	0.698	2	1.000	0.373
	ISI	329.654	***4.395***	***9.839***	***0.000***	***3.862***	***23.453***	***0.000***	***4.761***	***4.504***	***0.001***	3.446	3.260	0.017	1.044	0.339	0.571	***2.490***	***8.261***	***0.000***
	ISI x Genotype	329.654	8.791	1.392	0.192	7.725	0.487	0.859	9.523	1.155	0.322	6.892	0.511	0.824	2.088	0.456	0.644	4.980	0.271	0.928
	ISI x Sex	329.654	***4.395***	***3.648***	***0.005***	3.862	1.009	0.402	4.761	1.347	0.246	3.446	2.341	0.065	1.044	0.339	0.571	2.490	0.895	0.429
	ISI x Genotype x Sex	329.654	8.791	1.225	0.279	7.725	0.693	0.692	***9.523***	***2.624***	***0.005***	6.892	0.959	0.461	2.088	0.456	0.644	4.980	1.808	0.113
	Time bin	132.964	***1.773***	***536.121***	***0.000***	1.905	0.133	0.867	***1.562***	***32.651***	***0.000***	1.949	0.676	0.506	1.017	0.152	0.702	***1.650***	***5.285***	***0.010***
	Time bin x Genotype	132.964	3.546	0.512	0.705	3.810	1.060	0.377	3.124	1.150	0.333	3.898	1.294	0.276	2.035	0.685	0.510	3.300	0.957	0.422
	Time bin x Sex	132.964	1.773	1.186	0.305	1.905	0.247	0.771	1.562	0.655	0.485	1.949	2.962	0.056	1.017	0.152	0.702	1.650	1.012	0.354
	Time bin x Genotype x Sex	132.964	3.546	2.521	0.051	3.810	0.235	0.911	3.124	1.066	0.368	3.898	1.391	0.241	2.035	0.685	0.510	3.300	1.297	0.278
	ISI x Time bin	605.533	8.074	1.729	0.088	7.541	1.224	0.285	7.225	2.280	0.025	6.997	0.565	0.785	1.155	0.444	0.535	4.023	2.898	0.022
	ISI x Time bin x Genotype	605.533	16.148	1.060	0.391	15.082	1.002	0.452	14.451	1.102	0.352	13.994	1.253	0.233	2.311	0.412	0.693	8.045	1.322	0.231
	ISI x Time bin x Sex	605.533	8.074	1.272	0.255	7.541	0.518	0.833	7.225	0.636	0.731	6.997	0.819	0.571	1.155	0.444	0.535	4.023	1.345	0.253
	ISI x Time bin x Genotype x Sex	605.533	16.148	0.755	0.738	15.082	0.877	0.591	14.451	1.661	0.058	13.994	0.956	0.498	2.311	0.412	0.693	8.045	0.658	0.729
Trial x CS phase	**Trial**	291.832	***3.891***	***77.761***	***0.000***	***3.636***	***17.824***	***0.000***	***3.767***	***9.212***	***0.000***	4.193	1.711	0.144	1.449	5.281	0.013	***2.083***	***6.973***	***0.001***
	Trail x Genotype	291.832	7.782	0.636	0.743	7.272	0.475	0.858	7.535	0.769	0.623	8.386	2.331	0.017	2.899	0.472	0.696	4.166	1.131	0.345
	Trail x Sex	291.832	3.891	1.539	0.192	3.636	0.804	0.513	3.767	0.989	0.411	4.193	1.342	0.253	1.449	0.284	0.681	2.083	0.913	0.407
	Trail x Genotype x Sex	291.832	7.782	1.611	0.123	7.272	0.901	0.509	7.535	2.514	0.014	8.386	0.606	0.780	2.899	1.002	0.393	4.166	0.950	0.439

(*continued on next page*)

Table 1 (*continued*)

		Immobility - acquisition			Immobility - extinction			Rearing - acquisition			Rearing - extinction			Grooming - acquisition			Grooming - extinction		
	df error	**df**	**F**	***p***	**df**	**F**	***p***	**df**	**F**	***p***	**df**	**F**	***p***	**df**	**F**	***p***	**df**	**F**	***p***
CS phase	137.206	***1.829***	***365.065***	***0.000***	***1.798***	***15.834***	***0.000***	***1.629***	***15.870***	***0.000***	1.838	2.674	0.077	1.185	5.233	0.019	***1.680***	***5.168***	***0.010***
CS phase x Genotype	137.206	3.659	0.443	0.761	3.596	1.421	0.234	3.258	1.624	0.183	3.675	1.569	0.190	2.371	0.393	0.813	3.360	0.643	0.606
CS phase x Sex	137.206	1.829	0.619	0.526	1.798	0.790	0.444	1.629	0.523	0.557	1.838	2.753	0.072	1.185	1.012	0.331	1.680	2.246	0.119
CS phase x Genotype x Sex	137.206	3.659	1.818	0.135	3.596	0.615	0.636	3.258	1.623	0.184	3.675	1.787	0.140	2.371	1.135	0.332	3.360	0.541	0.675
Trial x CS phase	632.570	***8.434***	***32.906***	***0.000***	7.565	2.138	0.034	***6.605***	***22.848***	***0.000***	6.059	2.688	0.014	1.854	3.958	0.024	3.648	1.607	0.178
Trial x CS phase x Genotype	632.570	16.869	1.134	0.317	15.130	0.804	0.675	13.210	1.542	0.097	12.118	1.081	0.374	3.707	0.404	0.791	7.296	1.428	0.191
Trial x CS phase x Sex	632.570	8.434	1.500	0.149	7.565	0.479	0.863	6.605	0.483	0.837	6.059	1.966	0.068	1.854	0.574	0.552	3.648	0.532	0.696
Trial x CS phase x Genotype x Sex	632.570	16.869	1.081	0.368	15.130	0.834	0.640	***13.210***	***2.417***	***0.003***	12.118	0.918	0.529	3.707	0.963	0.426	7.296	1.016	0.421

rearing behavior (Fig. 7A, B, C, D).

Genotype and sex had a moderate impact on this association between 22-kHz USV emission and overt behaviors, most notably immobility ($F_{2,28} = 1.687$, $p = .203$; S: $F_{1,28} = 1.181$, $p = .286$; GxS: $F_{2,28} = 3.939$, $p = .031$). While the proportion of 22-kHz USV emitted during phases of immobility was particularly high in $SERT^{+/+}$ and $SERT^{+/-}$ females compared to $SERT^{+/+}$ and $SERT^{+/-}$ males, this was not evident in $SERT^{-/-}$ rats. An inverted pattern was seen for the association between 22-kHz USV emission and other motor activity ($F_{2,28} = 1.653$, $p = .210$; S: $F_{1,28} = 1.909$, $p = .178$; GxS: $F_2 = 4.120$, $p = .027$). The association between 22-kHz USV emission and rearing behavior was not affected by genotype and sex ($F_{2,28} = 0.017$, $p = .983$; S: $F_{1,28} = 1.843$, $p = .185$; GxS: $F_{2,28} = 0.141$, $p = .869$).

In line with the temporal association between the emission of 22-kHz USV and immobility within individuals, a positive correlation was found between total calling time and the time spent immobile across individuals ($r = 0.258$; $p = .020$). However, at the experimental group level, this positive correlation was only found in $SERT^{+/+}$ and $SERT^{+/-}$ females ($r = 0.531$; $p = .042$ and $r = 0.532$; $p = .013$; respectively; all other $p < .050$; Fig. 8A, A'; for representative ethograms, see Fig. 8B). Of note, low-short 22-kHz USV were very rarely detected.

Besides 22-kHz USV, 50-kHz USV occurred, with highest numbers of 50-kHz USV during the initial phase of acquisition. The emission of 50-kHz USV was likewise affected by genotype ($chi^2_2 = 7.428$, $p = .024$). While 48% of $SERT^{+/+}$ rats vocalized ($N = 12$ out of $N = 25$), 85% of $SERT^{-/-}$ rats emitted 50-kHz USV ($N = 17$ out of $N = 20$). $SERT^{+/-}$ rats displayed an intermediate phenotype and 50-kHz USV occurred in 53% of cases ($N = 19$ out of $N = 36$). Moreover, the tendency to call was influenced by sex ($chi^2_1 = 7.936$; $p = .005$), with a higher propensity in female than male rats. While 72% of females vocalized ($N = 34$ out of $N = 47$), only 41% of males emitted 50-kHz USV ($N = 14$ out of N = 34).

During the initial phase of acquisition, the number of 50-kHz USV emitted was also affected by genotype (G: $F_{2,75} = 4.272$, $p = .017$), with $SERT^{-/-}$ rats emitting more calls than $SERT^{+/-}$ and $SERT^{+/+}$ littermate controls ($p = .050$ and $p = .004$, respectively; Fig. 9A). Sex had no effect (S: $F_{1,75} = 0.931$, $p = .338$; GxS: $F_{2,75} = 0.140$, $p = .870$). When restricting the analysis to rats emitting 50-kHz USV, i.e. excluding non-vocalizing rats, the number of 50-kHz USV did not differ between genotypes and sexes (G: $F_{2,43} = 1.972$, $p = .152$; S: $F_{1,43} = 0.130$, $p = .720$; GxS: $F_{2,43} = 0.830$, $p = .367$). A different pattern was evident during the conditioning phase. Here, sex had an effect (S: $F_{1,75} = 11.234$, $p = .001$) with females vocalizing more than males, but not genotype (G: $F_{2,75} = 2.944$, $p = .059$; GxS: $F_{2,75} = 0.417$, $p = .660$; Fig. 9B). This effect remained after excluding non-vocalizing rats (G: $F_{2,43} = 1.892$, $p = .163$; S: $F_{1,43} = 7.521$, $p = .009$; GxS: $F_{2,43} = 0.290$, $p = .593$).

In summary, SERT deficiency was associated with reduced emission of 22-kHz USV during acquisition, whereas 50-kHz USV emission was enhanced, particularly during the initial phase of acquisition. Moreover, 22-kHz USV emission was higher in male than female rats. While females typically emitted longer 22-kHz USV with higher levels of frequency modulation than males, bouts emitted by females contained less 22-kHz USV than bouts emitted by males.

3.4. Alarm 22-kHz USV – Extinction

During extinction on the third day, where no shock followed the CS presentation, only 3% of rats (N = 2 out of $N = 81$), N = 1 $SERT^{+/+}$ male and N = 1 $SERT^{+/-}$ male, emitted 22-kHz USV. Low-short 22-kHz USV were not detected during extinction.

While 22-kHz USV occurred rarely, a moderate number of 50-kHz USV occurred, particularly during the initial context phase. The emission of 50-kHz USV was again affected by genotype ($chi^2_2 = 0.014$, $p = .905$). While only 12% of $SERT^{+/+}$ rats vocalized (N = 3 out of N = 25), still 55% of $SERT^{-/-}$ rats emitted 50-kHz USV ($N = 11$ out of N = 20). $SERT^{+/-}$ rats displayed an intermediate phenotype and 50-kHz USV occurred in 22% of cases (N = 8 out of N = 36). However, the

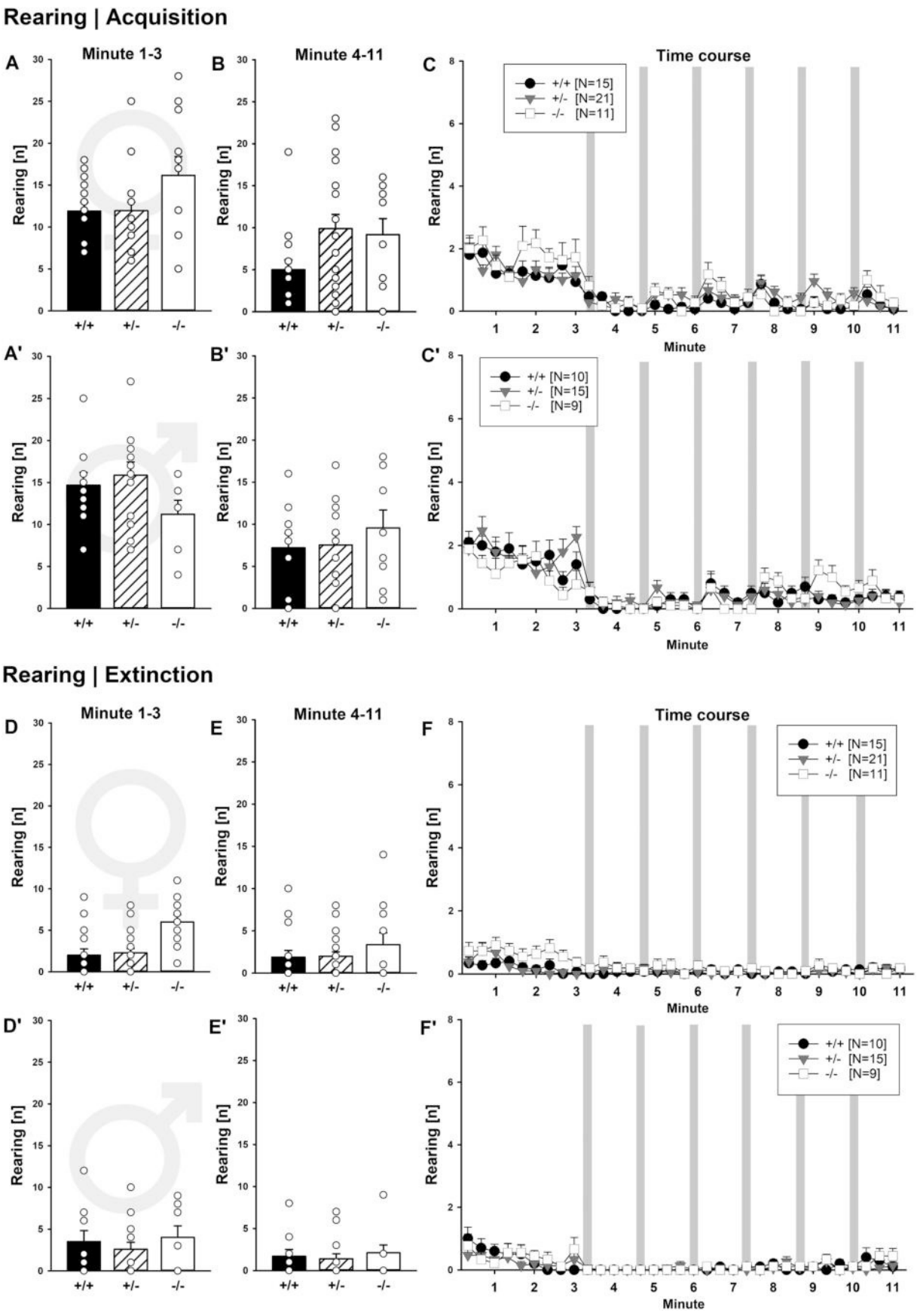


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Fig. 2. | Rearing.
Effects of SERT deficiency on rearing behavior during acquisition (A-C') and extinction (D-F'). Depicted is rearing behavior during the first three minutes of acquisition and extinction, respectively, for female (A, D) and male (A', D') $SERT^{+/+}$ (black bar), $SERT^{+/-}$ (striped bar), and $SERT^{-/-}$ (white bar) rats, followed by number of rearing behavior during the CS presentation period in minute 4–11 (B, B′; E, E') and the detailed time course of rearing in successive 20s time bins during acquisition (C, C′) and extinction (F, F′) for $SERT^{+/+}$ (black circle), $SERT^{+/-}$ (grey triangle), and $SERT^{-/-}$ (white square) rats. Grey bars represent the 20s time bins during CS presentation. During acquisition, CS presentation was followed by a mild electric shock. $N = 47$ female rats (15 +/+, 21 +/−, 11 −/−), $N = 34$ male rats (10 +/+, 15 +/−, 9 −/−). Data presented as mean ± SEM.

tendency to call was not influenced by sex ($chi^2_1 = 0.014$, $p = .905$). In fact, 28% of females ($N = 13$ out of N = 47) and 27% of males emitted 50-kHz USV ($N = 9$ out of N = 34).

During the initial context phase, the number of 50-kHz USV emitted was again affected by genotype (G: $F_{2,75} = 3.920$, $p = .024$), with $SERT^{-/-}$ rats emitting more calls than $SERT^{+/-}$ and $SERT^{+/+}$ littermate controls ($p = .015$ and $p = .005$, respectively; Fig. 9C). Sex had no effect (S: $F_{1,75} = 2.668$, $p = .107$; GxS: $F_{2,75} = 2.263$, $p = .111$). When restricting the analysis to rats emitting 50-kHz USV, i.e. excluding non-vocalizing rats, the number of 50-kHz USV did not differ between genotypes and sexes (G: $F_{2,16} = 0.756$, $p = .486$; S: $F_{1,16} = 1.136$, $p = .302$; GxS: $F_{2,16} = 1.377$, $p = .281$). During the cue phase, the emission of 50-kHz USV was low and genotype and sex had no effect (G: $F_{2,75} = 0.631$, $p = .535$; S: $F_{1,75} = 2.023$, $p = .159$; GxS: $F_{2,75} = 0.599$, $p = .552$; Fig. 9D).

In summary, SERT deficiency was associated with enhanced emission of 50-kHz USV during extinction, whereas 22-kHz USV emission was very low.

3.5. Body weight

Body weight during fear conditioning differed between genotypes and sexes, with most prominent genotype effects in males (G: $F_{2,75} = 11.269$, $p < .001$; S: $F_{2,75} = 668.319$, $p < .001$; GxS: $F_{2,81} = 3.960$, $p = .023$). $SERT^{-/-}$ rats were weighing less than $SERT^{+/-}$ and $SERT^{+/+}$ littermate controls ($p = .001$ and $p = .019$, respectively).

3.6. Hot plate

Pain sensitivity was not affected by genotype and sex and no differences in the latency to lift the first paw from the hot plate were found (G: $F_{2,81} = 0.337$, $p = .715$; S: $F_{1,81} = 3.527$, $p = .064$; GxS: $F_{2,81} = 0.224$, $p = .799$; Fig. 10).

4. Discussion

In the present experiment, we studied the effects of SERT deficiency on overt fear-related behavior and alarm 22-kHz USV during fear conditioning in male and female rats. While overt fear-related behavior was not affected by SERT deficiency and sex, the emission of alarm 22-kHz USV was clearly reduced in homozygous $SERT^{-/-}$ but not heterozygous $SERT^{+/-}$ mutants, as compared to their wildtype $SERT^{+/+}$ littermate controls. Genotype effects were particularly prominent in females. Females in general emitted fewer alarm 22-kHz USV than males.

4.1. Overt fear-related behavior

As expected, repeated tone-shock pairings during acquisition on the second day led to overt fear-related behavior, most notably behavioral inhibition. The locomotor pattern throughout acquisition also included escape-like reactions as an immediate response to the shock, followed by increasing levels of immobility up to the virtual absence of motor activity during CS presentations. Time spent immobile during acquisition, however, did not differ between genotypes and sexes. Likewise, no prominent effects of genotype and sex were found on rearing and grooming behavior. This indicates that acquisition of fear to an auditory CS was neither impaired nor enhanced in $SERT^{-/-}$ rats, in line with previous studies reporting no genotype differences during fear conditioning (Luoni et al., 2013; Schipper et al., 2018; Shan et al., 2018). However, it has to be noted that $SERT^{+/+}$ rats already displayed high levels of overt fear-related behavior, and this might have hampered the detection of further increases in $SERT^{-/-}$ rats.

During extinction on the third day, time spent immobile did also not differ between genotypes and sexes. This includes both the initial context phase of extinction, where no CS was presented yet, as well as the subsequent cue phase of extinction with CS presentations. Irrespective of genotype and sex, immobility gradually increased during the context phase, peaked at the beginning of the cue phase, and slowly decreased towards the end, reflecting intact extinction. The complete lack of genotype and sex differences despite a very detailed behavioral analysis indicates that SERT deficiency had no effect on overt fear-related behavior during extinction in the applied fear conditioning protocol (Borta et al., 2006). The lack of fear extinction deficits in $SERT^{-/-}$ rats might appear surprising in light of previous reports (Luoni et al., 2013; Nonkes et al., 2012; Schipper et al., 2011; Schipper et al., 2018; Schipper et al., 2019a; Schipper et al., 2019b; Shan et al., 2014; Shan et al., 2018). However, in contrast to the present study, fear extinction was typically assessed in a completely novel environment in such previous studies to exclude contextual memory components. Moreover, reported fear extinction deficits in $SERT^{-/-}$ rats primarily occurred either in studies with extensive extinction sessions or during extinction retrieval (Luoni et al., 2013; Nonkes et al., 2012; Schipper et al., 2011; Schipper et al., 2018; Schipper et al., 2019a; Schipper et al., 2019b; Shan et al., 2014; Shan et al., 2018). Therefore, future experiments might benefit from assessing fear extinction during exposure to the conditioning context versus a novel environment and from including a test phase for extinction retention. Moreover, although fear extinction deficits in $SERT^{-/-}$ rats were repeatedly reported, it is important to note that this phenotype depends on a number of factors, such as diet (Schipper et al., 2011), housing cages (Shan et al., 2014), and distractors present during extinction (Nonkes et al., 2012). Developmental stage (Schipper et al., 2019a) and previous test experience (Schipper et al., 2018) also appear to play prominent modulatory roles.

4.2. Alarm 22-kHz USV

Rats emitted alarm 22-kHz USV primarily during acquisition in response to repeated tone-shock pairings. During extinction when only the CS was presented, 22-kHz USV occurred rarely. This pattern is consistent with previous studies applying the same fear conditioning protocol. Typically, 70–90% of rats emit 22-kHz USV during acquisition, but only 0–60% during extinction (Borta et al., 2006; Schwarting et al., 2007; Wöhr et al., 2005; Wöhr and Schwarting, 2008a; Wöhr and Schwarting, 2008b; Yee et al., 2012a; Yee et al., 2012b). Similar patterns were also observed when other fear conditioning protocols were applied. There as well, 22-kHz USV were primarily emitted in response to shock application, whereas rats vocalized less on testing days where no shock was given (Hegoburu et al., 2011; Kassai and Gyertyán, 2012; Koo et al., 2004). Strategies to boost 22-kHz USV during extinction applied in preclinical studies not only included omitting rats emitting low numbers of 22-kHz USV during training but also shock priming immediately before extinction (de Vry et al., 2004). During acquisition, 22-kHz USV emission was particularly high during ISIs between CS presentations. This is in line with a study by Jelen et al. (2003), showing that 22-kHz USV levels are high during ISIs but that CS presentation leads to an abrupt decrease in 22-kHz USV emission. Jelen et al. (2003)

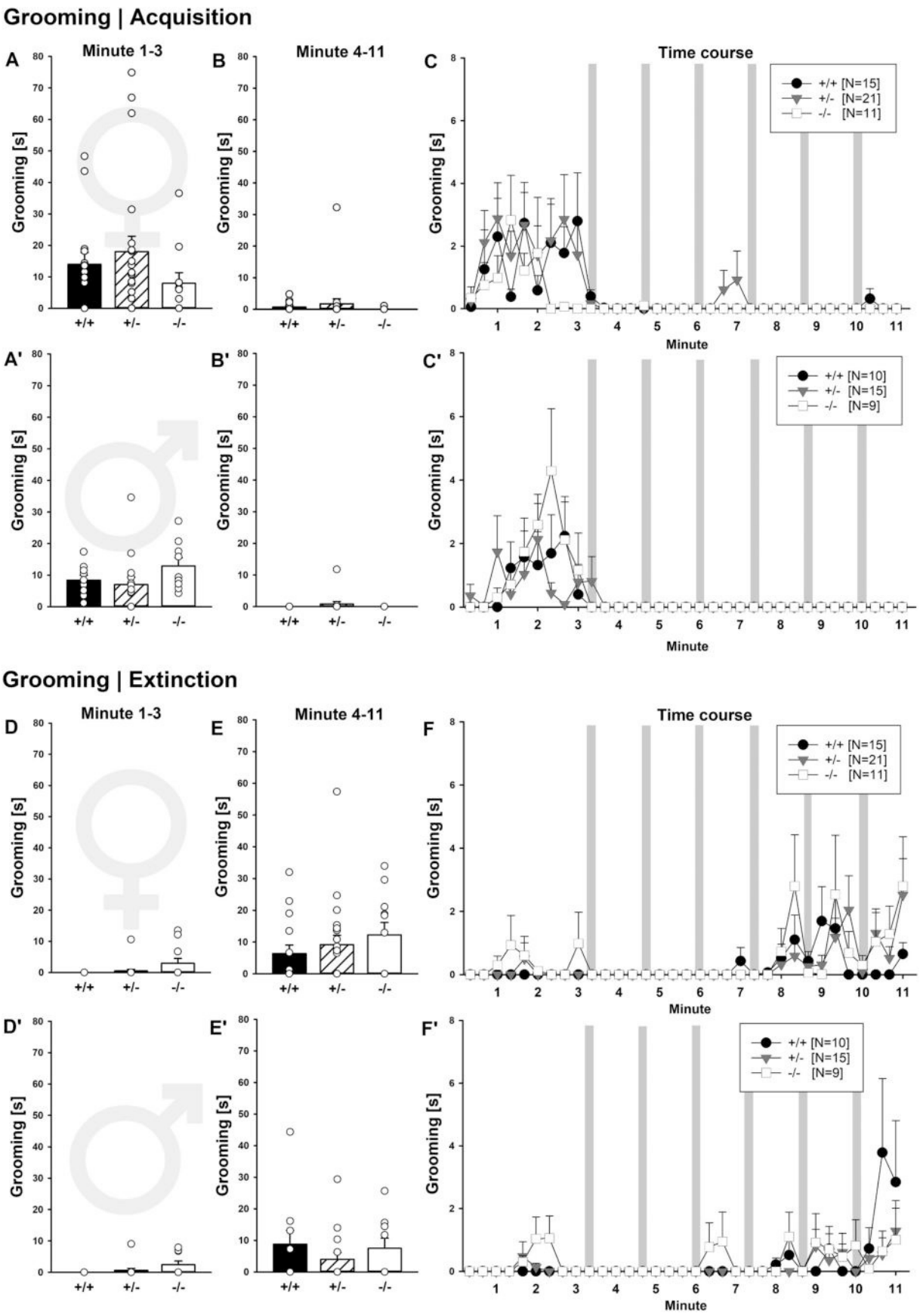

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Fig. 3. | Grooming.
Effects of SERT deficiency on the time spent grooming during acquisition (A-C′) and extinction (D-F′). Depicted is the time spent grooming during the first three minutes of acquisition and extinction, respectively, for female (A, D) and male (A', D') $SERT^{+/+}$ (black bar), $SERT^{+/-}$ (striped bar), and $SERT^{-/-}$ (white bar) rats, followed by total time spent grooming during the CS presentation period in minute 4–11 (B, B′; E, E') and the detailed time course of grooming in successive 20s time bins during acquisition (C, C′) and extinction (F, F′) for $SERT^{+/+}$ (black circle), $SERT^{+/-}$ (grey triangle), and $SERT^{-/-}$ (white square) rats. Grey bars represent the 20s time bins during CS presentation. During acquisition, CS presentation was followed by a mild electric shock. N = 47 female rats (15 +/+, 21 +/−, 11 −/−), N = 34 male rats (10 +/+, 15 +/−, 9 −/−). Data presented as mean ± SEM.

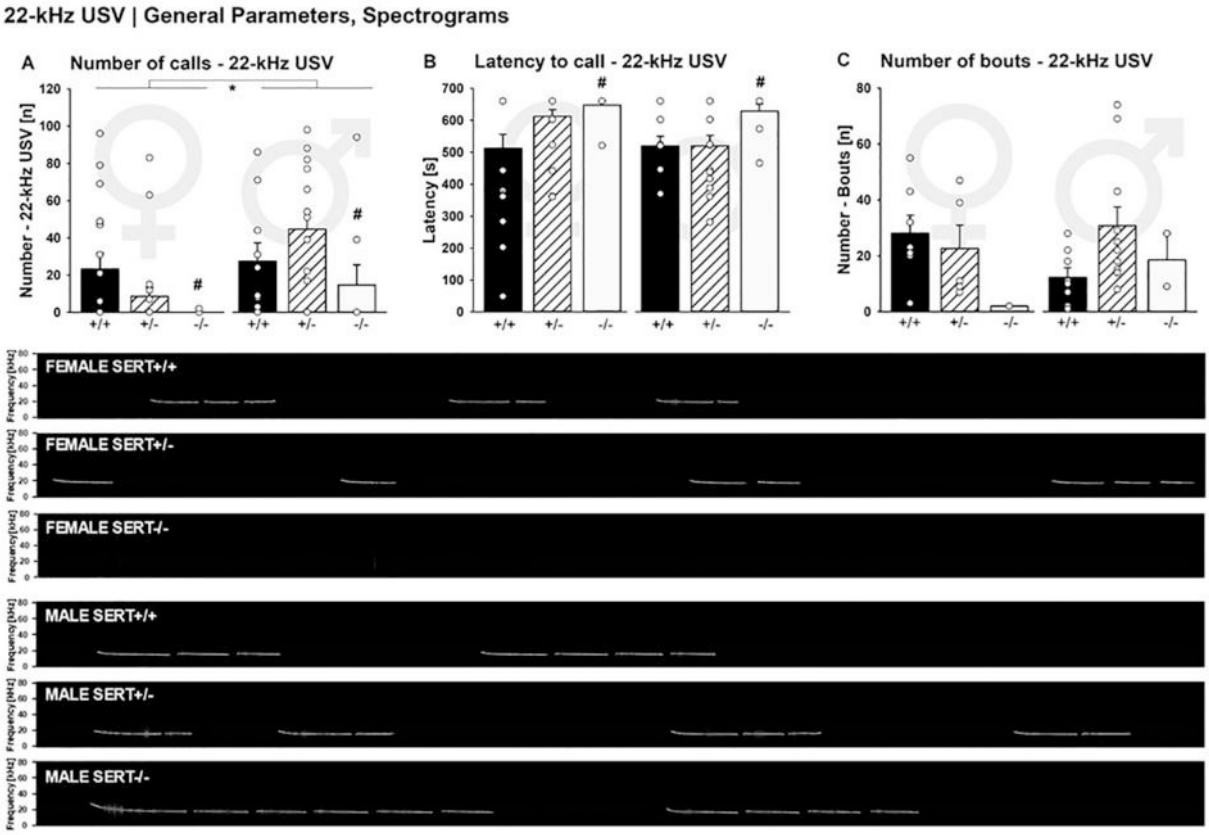

Fig. 4. | 22-kHz USV | General Parameters, Spectrograms.
Effects of SERT deficiency on the number of 22-kHz USV (A), the latency to emit 22-kHz USV (B), and the number of 22-kHz USV bouts (C) for female (left) and male (right) $SERT^{+/+}$ (black bar), $SERT^{+/-}$ (striped bar), and $SERT^{-/-}$ (white bar) rats during acquisition. N = 47 female rats (15 +/+, 21 +/−, 11 −/−), N = 34 male rats (10 +/+, 15 +/−, 9 −/−); for the number of 22-kHz USV bouts: *N* = 13 female rats (7 +/+, 5 +/−, 1 −/−), *N* = 21 male rats (8 +/+, 11 +/−, 2 −/−). Representative spectrograms depicting the bout structure of 22-kHz USV emission during the acquisition phase for each genotype and sex (D). Data presented as mean ± SEM. *$p < .05$ effect of sex, #$p < .05$ effect of genotype.

concluded that acute fear causes an immediate cessation of 22-kHz USV emission and speculate that this cessation might be part of an optimal behavioral strategy where 22-kHz USV are emitted to warn conspecifics in the absence of immediate danger but that its suppression by a signal of immediate danger may serve to reduce the risk of being detected, similar to behavioral inhibition.

4.3. Genotype differences in the production of alarm 22-kHz USV

SERT deficiency led to reduced 22-kHz USV emission during fear conditioning, consistent with the involvement of the 5-HT system in the production of alarm 22-kHz USV. In fact, SERT deficiency appears to be one of the factors responsible for the overall comparatively low number of vocalizing rats. While 60% of $SERT^{+/+}$ rats vocalized during acquisition, only 15% of $SERT^{-/-}$ rats emitted 22-kHz USV. Consistently, the number of 22-kHz USV emitted was also affected by genotype, with $SERT^{-/-}$ rats emitting fewer calls than $SERT^{+/-}$ and $SERT^{+/+}$ littermate controls. Reduced emission of 22-kHz USV in $SERT^{-/-}$ rats was, at least partly, driven by an increase in the latency to emit 22-kHz USV. Notably, most prominent genotype effects were evident during ISIs, while no differences during CS presentations were detected. $SERT^{-/-}$ rats spent less time emitting 22-kHz USV during ISIs than $SERT^{+/-}$ and $SERT^{+/+}$ littermate controls. This pattern was driven by a genotype- and sex-dependent change in calling behavior. While overall the average time spent calling per time bin was higher during ISIs than CS presentations, the tendency to vocalize more during ISIs, i.e. in the absence of CS presentations, was not seen in $SERT^{-/-}$ rats. In $SERT^{+/+}$ rats, in contrast, 22-kHz USV emission clearly increased during ISIs. Temporal organization and acoustic characteristics of 22-kHz USV were only mildly affected by genotype. It appears unlikely that the genotype differences in 22-kHz USV production are driven by differences in pain sensitivity because the behavioral response to thermal stimulation in the hot plate test was similar in all three genotypes, yet it might be advantageous to assess acute pain responses to electric shocks rather than thermal stimulation in future studies.

SERT deficiency in rats leads to a prominent increase in basal extracellular 5-HT levels (Homberg et al., 2007a). The reduction of alarm 22-kHz USV under conditions of constitutive increases in 5-HT

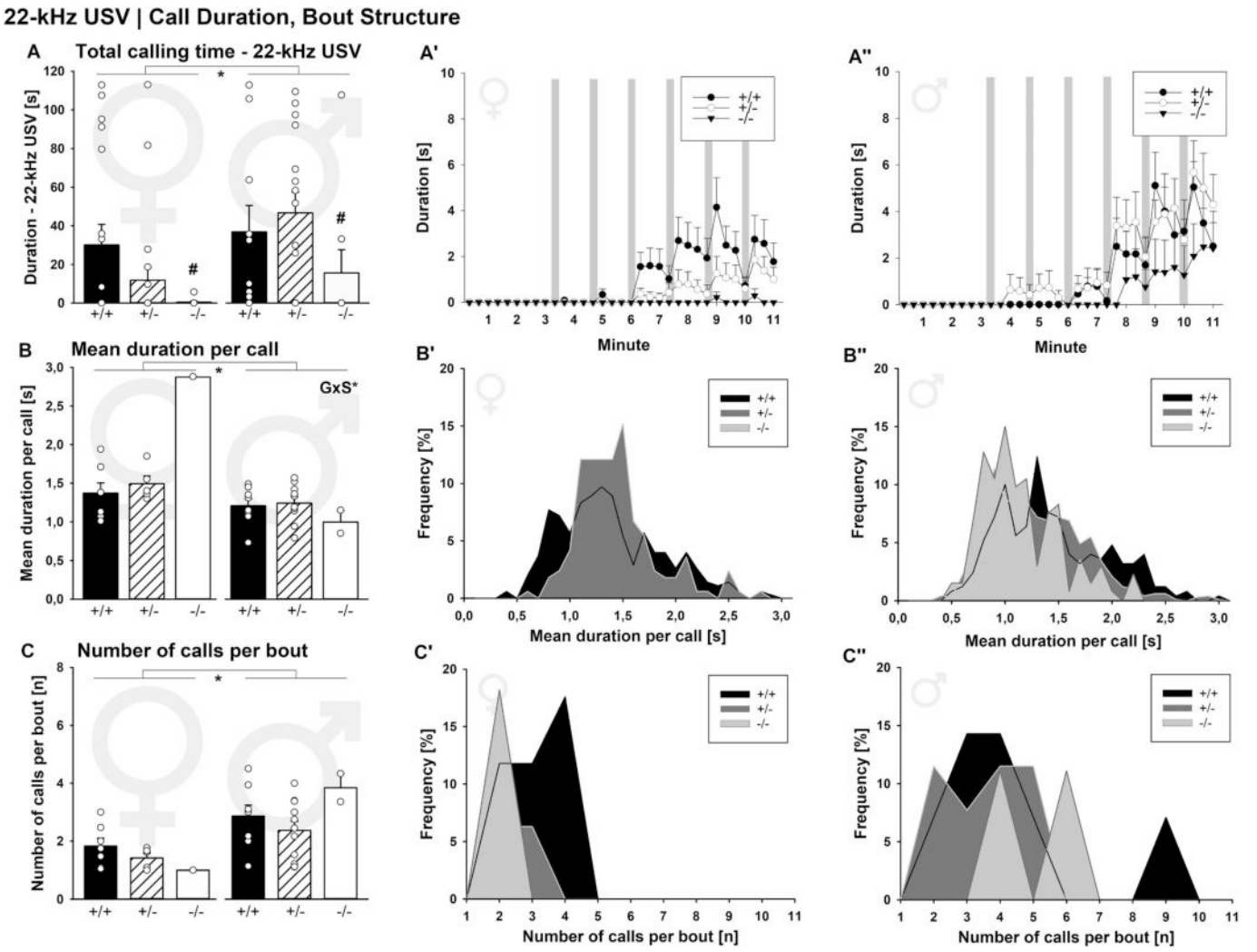

Fig. 5. | 22-kHz USV | Call Duration, Bout Structure.
Effects of SERT deficiency on total calling time (A) for female (left) and male (right) SERT$^{+/+}$ (black bar), SERT$^{+/-}$ (striped bar), and SERT$^{-/-}$ (white bar) rats during acquisition, followed by the detailed time course of total calling time in successive 20s time bins during acquisition for female (A') and male (A") SERT$^{+/+}$ (black circle), SERT$^{+/-}$ (grey triangle), and SERT$^{-/-}$ (white square) rats. Grey bars represent the 20s time bins during CS presentation. During acquisition, CS presentation was followed by a mild electric shock. N = 47 female rats (15 +/+, 21 +/−, 11 −/−), N = 34 male rats (10 +/+, 15 +/−, 9 −/−). Effects of SERT deficiency on mean call duration (B) and number of calls per bout (C) for females (left) and males (right) and area plots (B′–C′ for females, B"-C" for males, as percentage of all 22-kHz USV) depicting the frequency distribution in SERT$^{+/+}$ (black), SERT$^{+/-}$ (dark grey), SERT$^{-/-}$ (light grey); for number of calls per bout, only bouts with more than one call were included. N = 13 female rats (7 +/+, 5 +/−, 1 −/−), N = 21 male rats (8 +/+, 11 +/−, 2 −/−). Data presented as mean ± SEM. *p < .05 effect of sex, #p < .05 effect of genotype, GxS* p < .05 interaction between genotype and sex.

levels is in line with studies on the effects of acute 5-HT increases. As summarized by Sánchez (2003), all major SSRIs antagonize the emission of alarm 22-kHz USV. This includes citalopram, escitalopram, fluoxetine, fluvoxamine, paroxetine, and sertraline, although the inhibitory effect of fluoxetine is relatively mild, possibly because of notable 5-HT2 receptor antagonistic properties. In fact, 5-HT2 receptor agonists, such as DOI, were reported to block 22-kHz USV emission (de Vry et al., 1993). Citalopram exerted a biphasic dose-response effect, while escitalopram completely blocked alarm 22-kHz USV (Sánchez and Meier, 1997). Paroxetine is also particularly potent (Sánchez and Meier, 1997). Importantly, in contrast to SSRIs, other antidepressants that are less selective for SERT, such as clomipramine, imipramine, and reboxetine, have no or relatively mild inhibitory effects on the emission of alarm 22-kHz USV.

Another major change in the 5-HT system of SERT$^{-/-}$ rats is the desensitization of the 5-HT1A receptor (Homberg et al., 2008; Olivier et al., 2008a). Full or partial 5-HT1A receptor agonists were consistently reported to block alarm 22-kHz USV (Sánchez, 2003; Wöhr and van Gaalen, 2018). For instance, de Vry et al. (1993) demonstrated that 8-OH-DPAT, buspirone, gepirone, and ipsapirone, suppressed alarm 22-kHz USV and inhibitory effects of 5-HT1A receptor agonists were confirmed in more recent studies (de Vry et al., 2004; Kassai and Gyertyán, 2012). This indicates that under normal conditions 22-kHz USV emission is inhibited through the 5-HT1A receptor. However, when the 5-HT1A receptor is desensitized as in the SERT$^{-/-}$ rats, inhibition is weakened and one might therefore expect more 22-kHz USV. In the present study, however, decreased but not increased emission of 22-kHz USV was seen in SERT$^{-/-}$ rats and for this reason the 5-HT1A desensitization is unlikely to explain the genotype difference. Of note, SERT deficiency in rats does not seem to cause major alterations in other neurotransmitter systems (Homberg et al., 2007a) and persistent modifications in brain neurochemistry beyond 5-HT thus do not appear to underlie the reduction in 22-kHz USV emission.

Little is known about ultrasonic communication in SERT$^{-/-}$ rats. In a recent study, it was reported that the propensity of SERT$^{-/-}$ rats to emit 50-kHz USV but not 22-kHz USV in an open field was lower than in

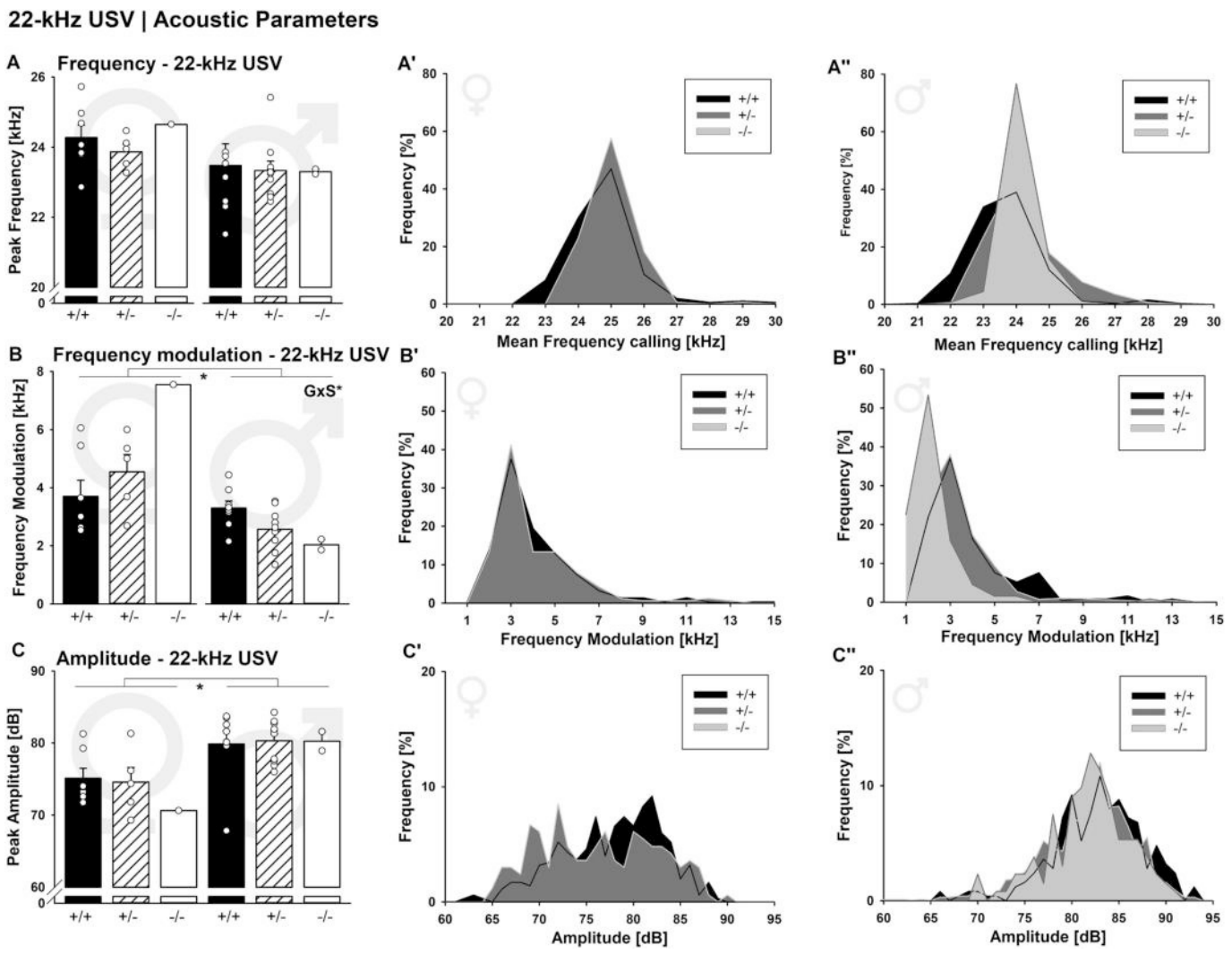

Fig. 6. | 22-kHz USV | Acoustic Parameters.
Effects of SERT deficiency on peak frequency (A), frequency modulation (B), and peak amplitude (C) of 22-kHz USV emitted by females (left) and males (right) and area plots (A'–C' for females, A"-C" for males, as percentage of all 22-kHz USV) depicting the frequency distribution in $SERT^{+/+}$ (black), $SERT^{+/-}$ (dark grey), and $SERT^{-/-}$ (light grey) rats. N = 13 female rats (7 +/+, 5 +/−, 1 −/−), N = 21 male rats (8 +/+, 11 +/−, 2 −/−). Data presented as mean ± SEM. *p < .05 effect of sex, GxS* p < .05 interaction between genotype and sex.

$SERT^{+/+}$ littermates, albeit the number of 50-kHz USV did not differ between genotypes (Golebiowska et al., 2019). Likewise, genotypes did not differ in the number of 50-kHz USV during reciprocal social interactions in adulthood. A detailed 50-kHz USV subtype analysis, however, revealed that subtype prevalence was mildly affected by genotype (Golebiowska et al., 2019). This is interesting because, in the present study, 50-kHz USV occurred particularly during the initial phase of acquisition and their emission was affected by genotype, with $SERT^{-/-}$ rats emitting more 50-kHz USV than $SERT^{+/-}$ and $SERT^{+/+}$ littermate controls. While 48% of $SERT^{+/+}$ rats vocalized during acquisition, 85% of $SERT^{-/-}$ rats emitted 50-kHz USV. Moreover, a moderate number of 50-kHz USV occurred particularly during the initial context phase of extinction and their emission was likewise affected by genotype, with $SERT^{-/-}$ rats again emitting more 50-kHz USV than $SERT^{+/-}$ and $SERT^{+/+}$ littermate controls. While only 12% of $SERT^{+/+}$ rats vocalized during extinction, still 55% of $SERT^{-/-}$ rats emitted 50-kHz USV. It was recently shown that 50-kHz USV emission can be modulated by 5-HT compounds, such as the 5-HT1A receptor agonist 8-OH-DPAT (Sadanada et al., 2012) and the 5-HT2C ligands CP 809,101 and SB 242084 (Wöhr et al., 2015). During fear conditioning, 50-kHz USV are rarely studied and little is known about their occurrence and function. Similar to the present study, 50-kHz USV emission was inhibited by shock application and inversely related to the production of 22-kHz USV in one study applying the same fear conditioning protocol (Yee et al., 2012a), in line with the idea that they reflect opposite affective states.

Another recent study reported effects of SERT availability on isolation-induced 40-kHz USV in rat pups (Houwing et al., 2019). Such 40-kHz USV are believed to reflect anxiety (Winslow and Insel, 1991) and it was found that male $SERT^{+/-}$ pups emitted more 40-kHz USV in response to isolation from mother and nest than male $SERT^{+/+}$ littermates. Moreover, perinatal fluoxetine treatment led to a prominent reduction in pup ultrasonic calling, particularly in male $SERT^{+/+}$ pups. $SERT^{-/-}$ rats were not tested.

In light of the anxiety phenotype previously reported in $SERT^{-/-}$ rats (Golebiowska et al., 2019; Olivier et al., 2008b; Schipper et al., 2011), the reduction in alarm 22-kHz USV might seem surprising because it is believed that 22-kHz USV reflect a negative affective state akin to anxiety and fear (Brudzynski, 2019). In line with the affective state hypothesis, inter-individual differences in anxiety-related behavior on the elevated plus maze were found to be positively associated with the emission of 22-kHz USV during fear conditioning (Borta et al., 2006) and one might thus have expected increased but not decreased emission of

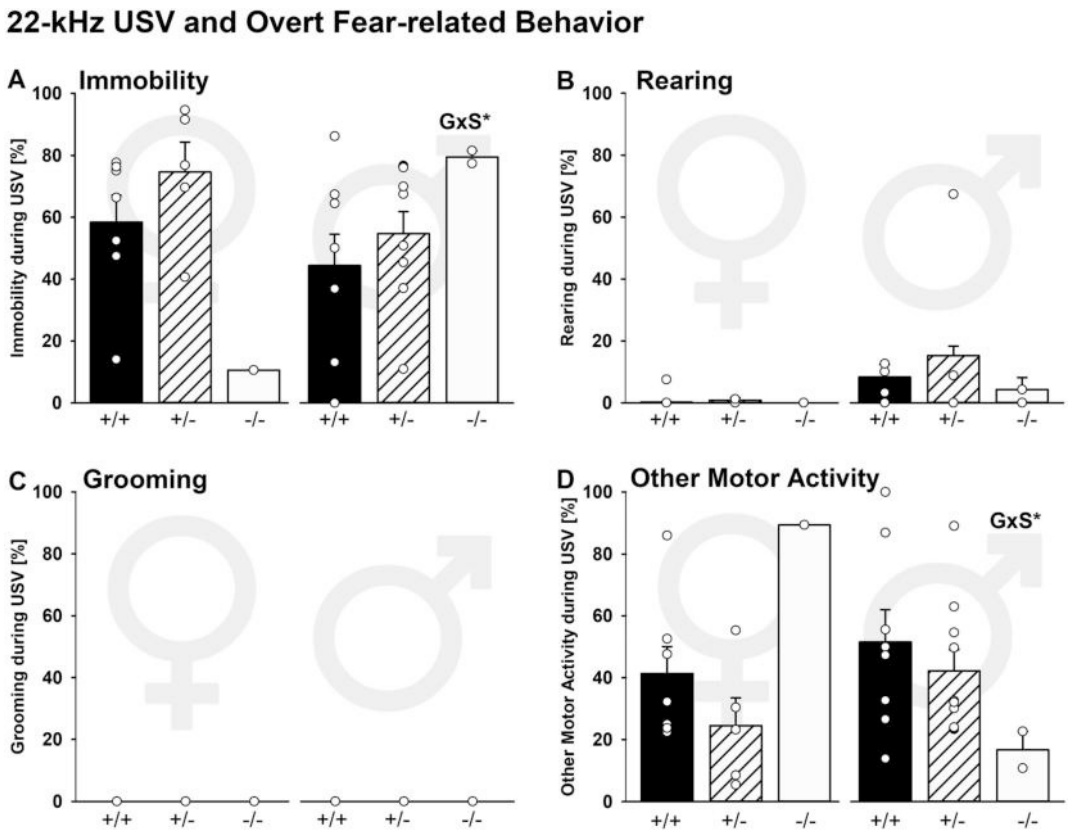

Fig. 7. | 22-kHz USV and Overt fear-related Behavior.
Effects of SERT deficiency on the proportion of specific behaviors, i.e. immobility (A), rearing (B), grooming (C), and other motor activity (D), while emitting 22-kHz USV for female (left) and male (right) $SERT^{+/+}$ (black bar), $SERT^{+/-}$ (striped bar), and $SERT^{-/-}$ (white bar) rats during acquisition. N = 13 female rats (7 +/+, 5 +/−, 1 −/−), N = 21 male rats (8 +/+, 11 +/−, 2 −/−). Data presented as mean ± SEM. GxS* $p < .05$ interaction between genotype and sex.

22-kHz USV in $SERT^{-/-}$ rats. However, 22-kHz USV emission is also believed to serve important communicative functions as alarm calls to warn conspecifics about threats (Blanchard et al., 1991; Blanchard et al., 1992). The observation that 22-kHz USV do not occur in response to the exposure of a predator in the absence of an audience, i.e. depends on the presence of conspecifics (Blanchard et al., 1991), is in line with the communicative function hypothesis and suggests that 22-kHz USV are, at least partly, independently regulated from anxiety or fear and as socially mediated alarm calls do not simply express a negative affective state. In fact, the social interpretation of the current situation seems to be a major determinant of 22-kHz USV emission besides anxiety and fear.

Interestingly, 5-HT is heavily involved in regulating social behavior and SERT deficiency was found to result in social deficits (Kiser et al., 2012). In juvenile rats, social play behavior was markedly reduced in male $SERT^{-/-}$ rats, including all major components, such as pinning, pouncing, and boxing, albeit following behavior was enhanced (Homberg et al., 2007c). Moreover, in adult rats, time spent in social contact was found to be lower during reciprocal social interactions in male $SERT^{-/-}$ rats, although they displayed more following behavior (Golebiowska et al., 2019). In the three-chambered social approach task, sociability was not altered but there was evidence for social recognition deficits obtained (Golebiowska et al., 2019). In a resident-intruder test, male $SERT^{-/-}$ rats displayed less aggressive behavior, with no alterations in other social behaviors (Homberg et al., 2007b). Finally, female $SERT^{-/-}$ rats spent less time in social contact and showed more self-grooming behavior during reciprocal social interactions in adulthood (Schipper et al., 2011). Together, this shows that social behavior is impaired in $SERT^{-/-}$ rats, possibly due to alterations in the social interpretation of the environment. Reduced emission of alarm 22-kHz USV in $SERT^{-/-}$ rats might therefore not be linked to genotype differences in anxiety and fear, in line with the lack of genotype differences in overt fear-related behavior. Reduced emission of alarm 22-kHz USV might rather reflect social deficits and it would be interesting to see whether social information processing is affected by SERT deficiency, for instance by measuring behavioral responses to 22-kHz USV in playback experiments (Brudzynski and Chiu, 1995; Fendt et al., 2018).

4.4. Sex differences in the production of alarm 22-kHz USV

The production of 22-kHz USV differed between sexes. During acquisition, the tendency to call was influenced by sex, with a higher propensity in male than female rats. In previous studies applying the same fear conditioning protocol, only males were studied and 70–90% of rats were found to emit 22-kHz USV during acquisition (Borta et al., 2006; Schwarting et al., 2007; Wöhr et al., 2005; Wöhr and Schwarting, 2008a; Wöhr and Schwarting, 2008b; Yee et al., 2012a; Yee et al., 2012b). In the present study, 62% of males vocalized, but only 28% of females emitted 22-kHz USV, indicating that not only SERT deficiency but also sex contributes to the overall comparatively low number of vocalizing rats. Females also emitted fewer 22-kHz USV and spent less time calling than their male conspecifics. Sex differences were particularly prominent during ISIs between CS presentations. Sex did not affect the latency to emit 22-kHz USV, indicating that the lower number of 22-kHz USV is due to a reduced repetition rate, i.e. less 22-kHz USV per time bin. Because males and females tended to differ in their behavioral response to thermal stimulation in the hot plate test one might speculate that sex differences in 22-kHz USV production are associated with differences in pain sensitivity. However, pain sensitivity tended to be higher in females than males and one thus would expect increased but

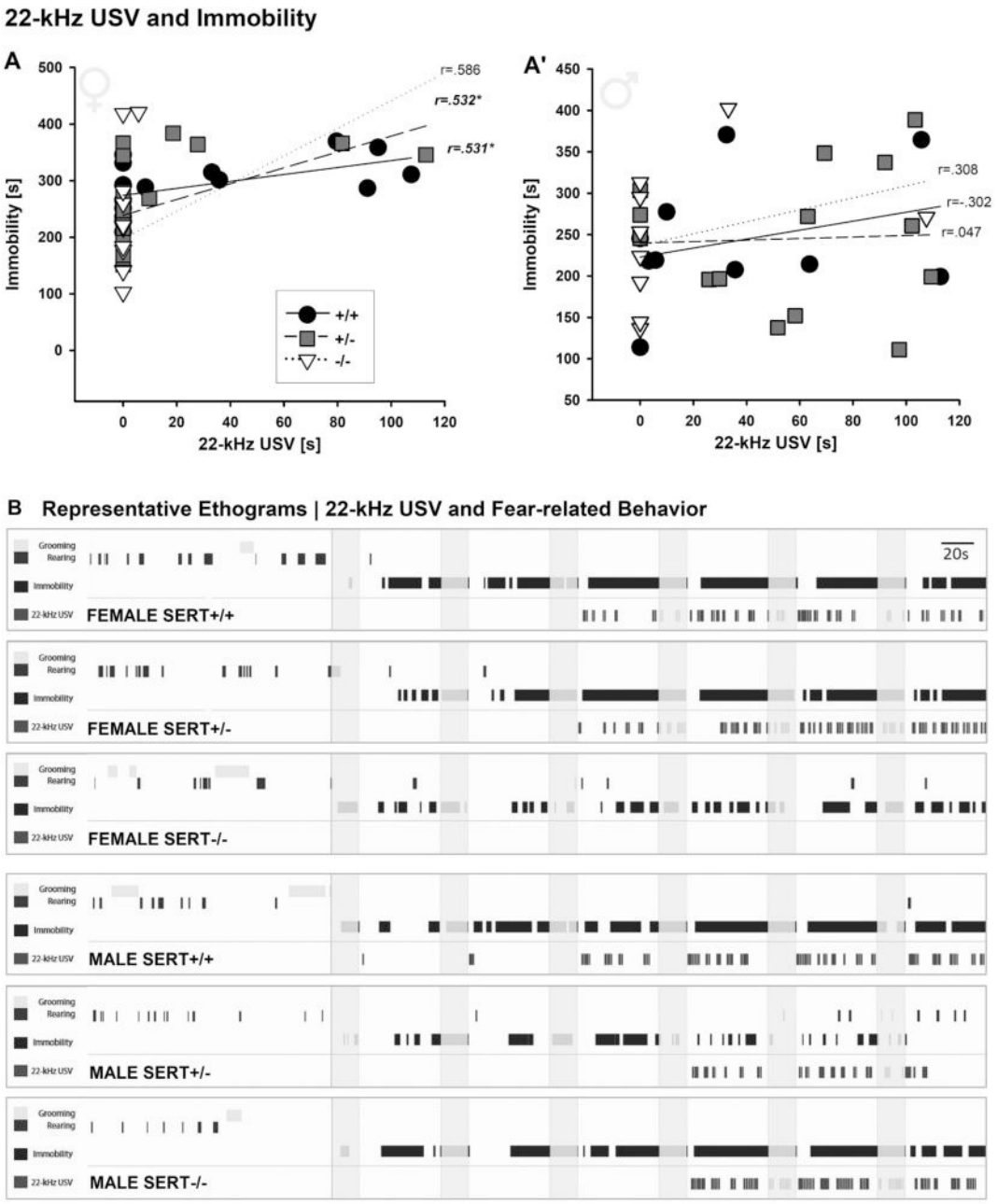

Fig. 8. | 22-kHz USV and Immobility.
Effects of SERT deficiency on the duration of immobility in relation to total calling time (A-A') for female (left) and male (right) $SERT^{+/+}$ (black circle), $SERT^{+/-}$ (grey square), $SERT^{-/-}$ (white triangle) rats. N = 47 female rats (15 +/+, 21 +/−, 11 −/−), N = 34 male rats (10 +/+, 15 +/−, 9 −/−). Representative ethograms depicting 22-kHz USV and overt fear-related behavior displayed during the acquisition phase for each genotype and sex (B). Active behaviors, i.e. grooming and rearing, are represented in green colors, immobility in purple, 22-kHz USV in light red. Blue accentuation represents CS presentation followed by foot shock. *$p < .05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

not decreased 22-kHz USV production in females. It therefore appears unlikely that the sex differences in 22-kHz USV production are driven by differences in pain sensitivity. Moreover, sex effects on the emission of 22-kHz USV do not appear to be caused by differences in anxiety and fear because overt fear-related behavior was similar in males and females. The fact that sex differences in 22-kHz USV occur in absence of differences in overt fear-related behavior, such as behavioral immobility, further supports the view that 22-kHz USV emission and overt fear-related behavior are, at least partly, independently regulated, but that sex differences could be linked to the communicative function of alarm 22-kHz USV.

Reduced 22-kHz USV emission in females is in contrast to initial findings obtained in a series of experiments on sex differences in anti-predator defensive behavior assayed in a visible burrow system. In social groups of rats living under such semi-natural conditions, females produced clearly more 22-kHz USV when exposed to a cat and it was proposed from a game theory perspective that higher 22-kHz USV levels may be particularly useful in warning pups due to the longer association by the female with the offspring (Blanchard et al., 1990; Blanchard et al., 1992; Shepherd et al., 1992). In subsequent studies, however, females were reported to emit fewer 22-kHz USV when exposed to air puffs (Inagaki and Mori, 2015; Inagaki and Sato, 2016) or electric foot shocks during fear conditioning (de Vry et al., 1993; Graham et al., 2009; Kosten et al., 2005; Kosten et al., 2006). Because the level of 22-kHz USV

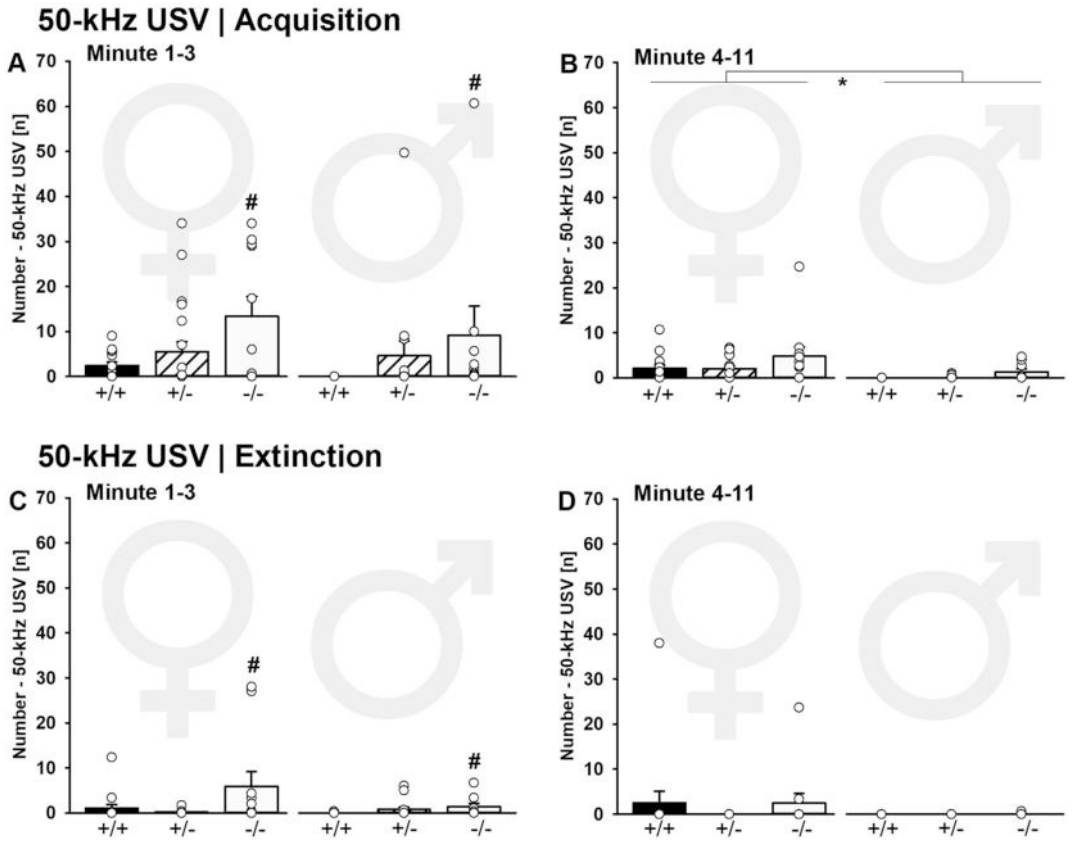

Fig. 9. | 50-kHz USV.
Effects of SERT deficiency on the number of 50-kHz USV during the initial phase of acquisition (A), the subsequent conditioning phase (B), the initial context phase of extinction (C), and the subsequent cue phase (D) for female (left) and male (right) $SERT^{+/+}$ (black bar), $SERT^{+/-}$ (striped bar), and $SERT^{-/-}$ (white bar) rats. N = 47 female rats (15 +/+, 21 +/−, 11 −/−), N = 34 male rats (10 +/+, 15 +/−, 9 −/−). Data presented as mean ± SEM. *p < .05 effect of sex, #p < .05 effect of genotype.

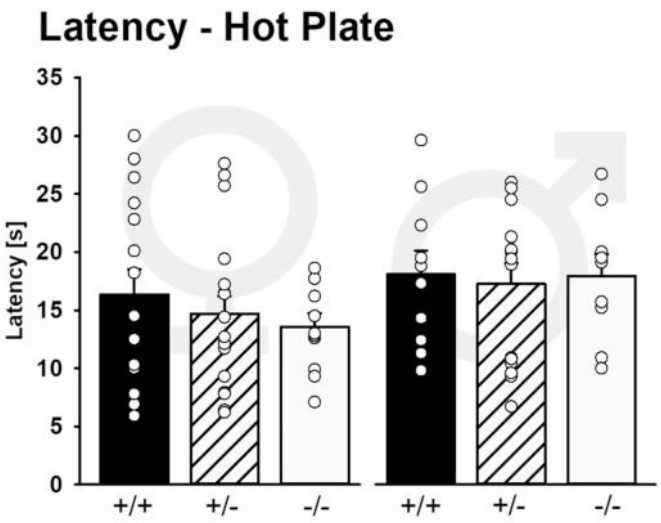

Fig. 10. | Hot Plate.
Effects of SERT deficiency on pain sensitivity in the hot plate test. Depicted is the latency to lift a paw for female (left) and male (right) $SERT^{+/+}$ (black bar), $SERT^{+/-}$ (striped bar), and $SERT^{-/-}$ (white bar) rats. N = 47 female rats (15 +/+, 21 +/−, 11 −/−), N = 34 male rats (10 +/+, 15 +/−, 9 −/−). Data presented as mean ± SEM.

emitted by males is lower following castration but can be restored by testosterone supplementation, it was suggested that sex differences in 22-kHz USV production are due to differences in testosterone concentrations (Inagaki and Mori, 2014). However, increasing testosterone concentrations in females to levels typically seen in males had no effect on 22-kHz USV emission (Inagaki and Mori, 2015).

In the present study, the temporal organization of 22-kHz USV emission was primarily dependent on sex. Prominent sex effects on mean call duration were evident and modulated by genotype, with females typically emitting longer 22-kHz USV than males[−−]. While the number of 22-kHz USV bouts was similar in males and females, bout length was strongly affected by sex, with bouts emitted by females containing less 22-kHz USV than bouts emitted by males. Because inter-call intervals between bouts are longer than within bouts, this change contributes to the slower repetition rate and thus the overall lower number of 22-kHz USV in females. Of note, this is again in contrast to the initial findings obtained in the visible burrow system during cat exposure, albeit a tendency for shorter call durations but longer bouts in females was not seen in all studies (Blanchard et al., 1992; Shepherd et al., 1992).

Moreover, in the present study, acoustic characteristics of 22-kHz USV differed between sexes. Specifically, 22-kHz USV emitted by males were louder than the ones emitted by females. Frequency modulation also differed between sexes and this sex effect was modulated by genotype. In general, 22-kHz USV emitted by females were characterized by higher levels of frequency modulation than the ones emitted by males. The sex difference was particularly prominent in $SERT^{-/-}$ rats. Peak frequency of 22-kHz USV, in contrast, did not differ between sexes, albeit a tendency for higher peak frequency in females was evident. While peak amplitude and frequency modulation were not assessed in

the visible burrow system, peak frequency was reported to be higher in females exposed to a cat (Blanchard et al., 1992; Shepherd et al., 1992). Peak frequency of 22-kHz USV was also higher in females exposed to air puffs, in the absence of sex differences in call duration and frequency modulation (Inagaki and Sato, 2016).

Together, the present finding of reduced 22-kHz USV emission in females is in contrast to studies where 22-kHz USV were evoked by cat exposure (Blanchard et al., 1990; Blanchard et al., 1992; Shepherd et al., 1992) but in line with studies using air puffs (Inagaki and Mori, 2015; Inagaki and Sato, 2016) or electric foot shocks (de Vry et al., 1993; Graham et al., 2009; Kosten et al., 2005; Kosten et al., 2006). A major difference between the studies reporting reduced versus enhanced 22-kHz USV emission in females is not only the stimulus but also the social environment. While sex differences in response to cat exposure were evaluated in the presence of an audience (Blanchard et al., 1990; Blanchard et al., 1992; Shepherd et al., 1992), there were no conspecifics present during the exposure to air puffs (Inagaki and Mori, 2015; Inagaki and Sato, 2016) or electric foot shocks (de Vry et al., 1993; Graham et al., 2009; Kosten et al., 2005; Kosten et al., 2006). Moreover, little is still known about sex differences in the acoustic features of 22-kHz USV and few data on 22-kHz USV recorded under standardized conditions in response to air puffs or electric foot shocks are available. It is currently unclear whether such sex differences simply reflect anatomical differences between males and females, such as body weight, lung capacity and vocal tract length (Hegoburu et al., 2011; Inagaki et al., 2012; Riede et al., 2020), or whether they serve communicative functions, for instance through providing additional features by which listening conspecifics may be able to determine the sex of the vocalizing rat, as suggested by Blanchard et al. (1992).

Of note, the emission of 50-kHz USV was likewise affected by sex during acquisition, with a higher propensity in female than male rats. While 72% of females vocalized, only 41% of males emitted 50-kHz USV. Higher 50-kHz USV emission in females was particularly prominent during the conditioning phase. During extinction, the tendency to emit 50-kHz USV was not influenced by sex and the number of 50-kHz USV emitted during the initial context phase and the subsequent cue phase was similar in males and females. Although no data on sex differences in 50-kHz USV emission during fear conditioning is available to our knowledge, higher 50-kHz USV levels in females were reported in response to an anesthetized conspecific (Blanchard et al., 1993) and stroking by an human experimenter (Kosten et al., 2005).

4.5. Alarm 22-kHz USV and overt fear-related behavior

By means of temporal analyses using high-resolution ethograms, the time-matched occurrence of 22-kHz USV and overt behaviors was determined and it was found that rats emitted 22-kHz USV primarily during phases of immobility. Specifically, rats emitted 57% of all 22-kHz USV during phases of immobility, whereas 2% occurred during rearing, and none during grooming. During other types of motor activity, typically locomotor activity, 41% of all 22-kHz USV were emitted. Particularly high levels of 22-kHz USV during immobility were also seen in other studies, where the relation of overt fear-related behavior and foot-shock induced 22-kHz USV during fear conditioning was investigated (Dupin et al., 2019; Hegoburu et al., 2011). Genotype and sex had a moderate impact on this association between 22-kHz USV emission and overt fear-related behaviors, most notably immobility. While the proportion of 22-kHz USV emitted during phases of immobility was particularly high in $SERT^{+/+}$ and $SERT^{+/-}$ females compared to $SERT^{+/+}$ and $SERT^{+/-}$ males, this was not evident in $SERT^{-/-}$ rats.

In line with the temporal association between the emission of 22-kHz USV and immobility within individuals, a positive correlation was found between total calling time and the time spent immobile across individuals, in line with previous studies (Wöhr et al., 2005; Wöhr and Schwarting, 2008a; Wöhr and Schwarting, 2008b; Yee et al., 2012b). However, at the experimental group level, this positive correlation was only found in $SERT^{+/+}$ and $SERT^{+/-}$ females. This dissociation again supports the view that the emission of 22-kHz USV does not simply express a negative affective state. Interestingly, there is evidence from neurotoxic lesion studies suggesting that basolateral amygdala lesions reduce immobility and 22-kHz USV similarly, whereas central amygdala lesions primarily affect 22-kHz USV (Koo et al., 2004; but see: Choi and Brown, 2003). Because in mouse studies SERT deficiency was reported to affect neuronal morphology in the amygdala (Wellman et al., 2007), it would be interesting to test whether similar effects are present in rats and whether this could explain the genotype differences in the emission of alarm 22-kHz USV in the absence of differences in overt fear-related behavior, most notably behavioral immobility.

5. Conclusion

SERT deficiency led to a clear reduction in the emission of alarm 22-kHz USV during fear conditioning. Overt fear-related behavior was not affected. This supports the view that 22-kHz USV are, at least partly, independently regulated from anxiety or fear and as socially mediated alarm calls do not simply express a negative affective state. Reduced 22-kHz USV emission in rats lacking SERT might be due to social deficits in the use of 22-kHz USV as a socio-affective signal to warn conspecifics about threats.

Author contributions

Wöhr designed the study and acquired funding. Willadsen and Wöhr performed the experiments, compiled the figures, analyzed the data, and wrote the manuscript. All authors read, critically revised, and approved the final manuscript.

Ethical statement

All procedures were conducted in 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 (Tierschutzbehörde, Regierungspräsidium Gießen, Germany).

Declaration of Competing Interest

The authors declare no competing interests.

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STUDIE IV

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Fear Extinction and Predictive Trait-Like Inter-Individual Differences in Rats Lacking the Serotonin Transporter

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Abstract: Anxiety disorders are associated with a failure to sufficiently extinguish fear memories. The serotonergic system (5-hydroxytryptamine, 5-HT) with the 5-HT transporter (5-HTT, SERT) is strongly implicated in the regulation of anxiety and fear. In the present study, we examined the effects of SERT deficiency on fear extinction in a differential fear conditioning paradigm in male and female rats. Fear-related behavior displayed during acquisition, extinction, and recovery, was measured through quantification of immobility and alarm 22-kHz ultrasonic vocalizations (USV). Trait-like inter-individual differences in novelty-seeking, anxiety-related behavior, habituation learning, cognitive performance, and pain sensitivity were examined for their predictive value in forecasting fear extinction. Our results show that SERT deficiency strongly affected the emission of 22-kHz USV during differential fear conditioning. During acquisition, extinction, and recovery, SERT deficiency consistently led to a reduction in 22-kHz USV emission. While SERT deficiency did not affect immobility during acquisition, genotype differences started to emerge during extinction, and during recovery rats lacking SERT showed higher levels of immobility than wildtype littermate controls. Recovery was reflected in increased levels of immobility but not 22-kHz USV emission. Prominent sex differences were evident. Among several measures for trait-like inter-individual differences, anxiety-related behavior had the best predictive quality.

Keywords: fear conditioning; freezing; ultrasonic vocalizations; alarm calls; novelty-seeking; anxiety; cognition; pain; SERT; 5-HTT

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1. Introduction

Excessive anxiety and fear are hallmarks of a number of neuropsychiatric disorders, most notably anxiety disorders, including phobias and post-traumatic stress disorder (PTSD) [1]. It is thought that such neuropsychiatric disorders are associated with a failure

to sufficiently extinguish fear memories [2]. Fear extinction is the inhibition of conditioned fear responses that is normally seen as a consequence of repeated exposure to a conditioned stimulus (CS) in the absence of an aversive unconditioned stimulus (UCS) [3]. It is thus a key component of the widely applied exposure therapy in the treatment of excessive anxiety and fear [4]. Limiting the efficacy of exposure therapy, however, stress and other environmental factors can inhibit fear extinction [5]. Moreover, there is a large variability between individuals and personality traits appear to play a prominent modulatory role [6]. Although recent efforts helped to dissect neurobiological mechanisms underlying fear extinction [7], little is still known about neurobiological factors associated with personality traits modulating fear extinction.

A prime candidate for explaining a significant proportion of the inter-individual differences seen during fear extinction is the serotonergic system (5-hydroxytryptamine, 5-HT). The 5-HT system fulfills a wide variety of functions and is strongly implicated in the regulation of anxiety and fear [8–10]. For instance, it is closely associated with anxiety disorders, including PTSD [11,12]. A key component of the 5-HT system is the 5-HT transporter (5-HTT, SERT), which regulates 5-HT availability in the synaptic cleft and terminates 5-HT signaling through reuptake of 5-HT into the presynaptic terminal [13].

In humans, the polymorphic region in the promoter of the SERT gene *SLC6A4* (5-HTTLPR) leads to the formation of two major variations, a short and a long allelic variant [14]. The short allelic variant leads to reduced transcription and altered function of SERT, translating into elevated levels of neuroticism, which includes higher anxiety levels [15], and increased acquisition [16] followed by reduced extinction [17] of fear. At the neurobiological level, such traits are paralleled by anatomical and functional correlates in multiple brain regions, including stronger amygdala activation in response to fearful stimuli [18]. Together, this contributes to a greater risk for suffering from PTSD after stressful life events [19].

By partially or fully reducing SERT expression through genetic modification, the consequences of limited SERT availability can be modelled in mice and rats [20,21]. In rats, the complete absence of SERT leads to a prominent increase in basal extracellular 5-HT levels and unresponsiveness to the selective 5-HT reuptake inhibitor citalopram [21]. Similar to human carriers of the short allelic variant, anxiety-related behavior is enhanced in rats lacking SERT. This is observable in unconditioned tasks, such as open field, elevated plus maze, light-dark test, and novelty suppressed feeding [22–26].

Fear extinction can be studied in rats [27] and SERT availability was found to play a modulatory role [28]. While acquisition of fear-related behavior towards threat signaling stimuli appears not to be affected in the majority of studies, the ability to extinguish fear-related behavior was repeatedly found to be impaired [23,25,29–35]. Furthermore, lack of SERT impedes extinction recall by means of recovery of formerly extinguished fear-related immobility [32,34]. Evidence for a conserved role of SERT in fear extinction was also obtained in mouse studies [36].

We have recently applied a fear conditioning paradigm in rats lacking SERT and obtained evidence for strong effects on fear-related behavior by measuring alarm calls in addition to immobility [37]. Rats emit whistle-like calls in the ultrasonic range, so called ultrasonic vocalizations (USV) [38,39]. In aversive situations, long and low-frequency 22-kHz USV occur. Under natural conditions, they can be observed during aggressive interactions with conspecifics [40,41] and exposures to predators or their odors [42,43]. Standardized procedures to evoke them in the laboratory include the administration of air puffs [44–46], acoustic startle stimuli [47,48], and electric shocks [49,50]. The emission of 22-kHz USV is believed to reflect a negative affective state akin to anxiety and fear. In fact, 22-kHz USV serve as additional measures in fear conditioning experiments because they allow to reveal effects of experimental manipulations that the standard measure immobility fails to capture [51]. For instance, early life stressors, such as prenatal exposure to the viral mimic polyI:C, were found to enhance 22-kHz USV emission in absence of overt behavioral differences [52]. In our most recent study on the effects of SERT availability, we found

that rats lacking SERT emitted fewer 22-kHz USV than controls [37]. This effect was seen in absence of overt behavioral differences despite a detailed behavioral analysis and was found to be more prominent in females than in males [37].

This genotype effect is particularly relevant in the context of personality traits modulating fear extinction because the emission of 22-kHz USV is characterized by robust inter-individual differences. For instance, rats characterized by high levels of trait anxiety emit more 22-kHz USV when challenged with tone-shock pairings during fear conditioning [53]. Moreover, factors known to shape trait-like inter-individual differences were repeatedly associated with alterations in 22-kHz USV emission. This includes prenatal immune activation [52], maternal neglect [54], and juvenile stress exposure [55].

Together, fear extinction is affected by various factors, resulting in substantial variability between individuals. In the present study, we aimed at identifying trait-like inter-individual differences driving a significant proportion of the variability between individuals seen during fear extinction. To this aim, we examined the effects of SERT deficiency on fear extinction in a differential fear conditioning paradigm in male and female rats. During differential fear conditioning, one CS was repeatedly paired with electric foot shocks (CS+) but not the other (CS-). Fear-related behavior displayed during acquisition, extinction, and recovery, was measured through quantification of 22-kHz USV emission and immobility. Trait-like inter-individual differences in novelty-seeking, anxiety-related behavior, habituation learning, cognitive performance, and pain sensitivity were examined for their predictive value in forecasting fear extinction.

2. Results

2.1. Body Weight

SERT deficiency affected body weight in a sex-dependent manner (G: $F_{2,87} = 14.093$, $p < 0.001$; S: $F_{1,87} = 262.820$, $p < 0.001$; GxS: $F_{2,87} = 9.216$, $p < 0.001$). While no prominent genotype differences were evident in females (all $p > 0.05$, Figure 1A), SERT deficiency affected body weight in males, with male SERT$^{-/-}$ rats showing consistently lower body weights than SERT$^{+/-}$ and SERT$^{+/+}$ littermates (all $p < 0.001$, Figure 1B).

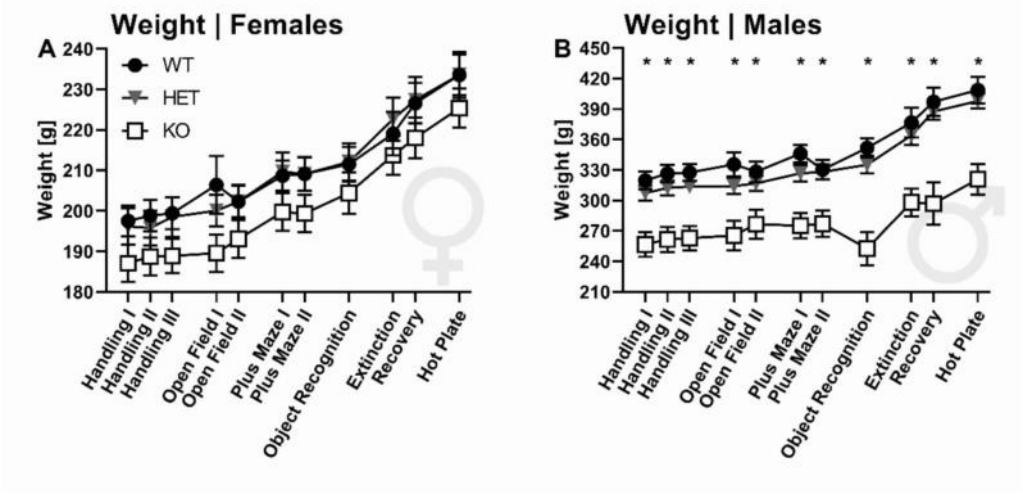

Figure 1. Body weight. Effects of SERT deficiency on body weight across different testing procedures for (**A**) female and (**B**) male SERT$^{+/+}$ (black circles), SERT$^{+/-}$ (grey triangle), and SERT$^{-/-}$ (white square) rats. N = 44 female rats (15 +/+, 15 +/−, 14 −/−), N = 43 male rats (14 +/+, 15 +/−, 14 −/−). Data are presented as mean ± SEM. * $p < 0.05$ effect of genotype, as compared to SERT$^{+/-}$ and SERT$^{+/+}$ rats.

2.2. Differential Fear Conditioning

2.2.1. 22-kHz USV Prevalence

When exposed to tone-shock pairings during the acquisition phase of the first day of differential fear conditioning, the emission of 22-kHz USV was strongly affected by genotype (G: $chi^2_2 = 7.970$, $p = 0.019$) and sex (S: $chi^2_2 = 20.077$, $p < 0.001$). While 52% ($N = 15$ out of $N = 29$) of $SERT^{+/+}$ and 47 % ($N = 14$ out of $N = 30$) of $SERT^{+/-}$ rats emitted 22-kHz USV during acquisition, 22-kHz USV emission rates were low in $SERT^{-/-}$ littermates and only 18% ($N = 5$ out of $N = 28$) vocalized (Figure 2A). Genotype differences appear to be driven by male rats. With 63 % ($N = 27$ out of $N = 43$) the majority of male rats emitted 22-kHz USV, whereas only 16 % ($N = 7$ out of $N = 44$) of females did (Figure 2A).

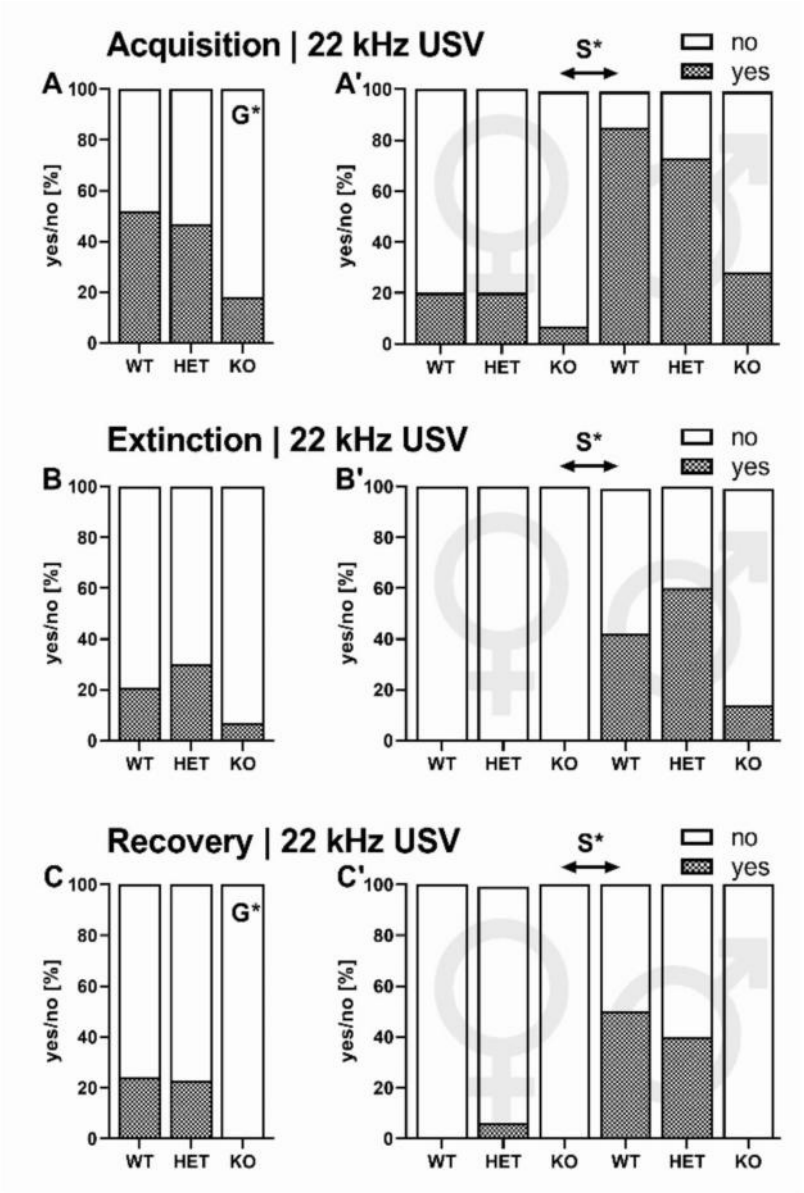

Figure 2. 22-kHz USV prevalence. Effects of SERT deficiency on the prevalence of 22-kHz emission (yes—checkered bar; no—transparent bar) during acquisition (**A**,**A′**), extinction (**B**,**B′**), and recovery (**C**,**C′**). Prevalence of 22-kHz USV is shown for $SERT^{+/+}$, $SERT^{+/-}$, and $SERT^{-/-}$ rats with sexes pooled (**A**,**B**,**C**) and separated by sex (**A′**,**B′**,**C′**), with females on the left and males on the right side of the panel. $N = 44$ female rats (15 +/+, 15 +/−, 14 −/−), N = 43 male rats (14 +/+, 15 +/−, 14 −/−). Data are presented as mean ± SEM. G* $p < 0.05$ effect of genotype, S* $p < 0.05$ effect of sex.

When challenged with CS presentations in another context during extinction on the second day, the emission of 22-kHz USV tended to differ between genotypes (G: $chi^2_2 = 4.849$, $p = 0.089$) and was strongly affected by sex (S: $chi^2_2 = 21.620$, $p < 0.001$). During extinction, 21% ($N = 6$ out of $N = 29$) of $SERT^{+/+}$ and 30% ($N = 9$ out of $N = 30$) of $SERT^{+/-}$ rats but only 7% ($N = 2$ out of $N = 28$) of $SERT^{-/-}$ littermates emitted 22-kHz USV (Figure 2B). Again, genotype effects were driven by male rats. With 40% ($N = 17$ out of $N = 43$) a large number of male rats emitted 22-kHz USV, whereas no female did ($N = 0$ out of $N = 44$, Figure 2B').

Seven days after extinction training, rats were reintroduced to the extinction context. Emission of 22-kHz USV was affected by genotype (G: $chi^2_2 = 7.925$, $p = 0.019$) and sex (S: $chi^2_2 = 12.591$, $p < 0.001$). While 24% ($N = 7$ out of $N = 29$) of $SERT^{+/+}$ and 23% ($N = 7$ out of $N = 30$) of $SERT^{+/-}$ rats emitted 22-kHz USV during recovery, no 22-kHz USV were detected in $SERT^{-/-}$ littermates ($N = 0$ out of $N = 28$, Figure 2C). Similar to acquisition and extinction, genotype effects were driven by male rats. When split into sexes, 30% ($N = 13$ out of $N = 43$) of male rats emitted 22-kHz USV, whereas only 2% ($N = 1$ out of $N = 44$) of females did (Figure 2C).

2.2.2. Overall Immobility and 22-kHz USV Total Calling Time

During acquisition, the overall time spent immobile was high in all experimental conditions irrespective of genotype (G: $F_{2,87} = 0.265$, $p = 0.768$, Figure 3A) and sex (S: $F_{1,87} = 0.135$, $p = 0.714$, GxS: $F_{2,87} = 0.563$, $p = 0.572$, Figure 3A'). Consistent with 22-kHz USV prevalence, however, the time spent emitting 22-kHz USV during acquisition was affected by genotype (G: $F_{2,87} = 4.688$, $p = 0.012$) and sex (S: $F_{1,87} = 25.538$, $p < 0.001$, GxS: $F_{2,87} = 2.818$, $p = 0.066$). $SERT^{-/-}$ rats spent less time calling compared to their $SERT^{+/-}$ and $SERT^{+/+}$ littermates ($p = 0.014$ and $p = 0.008$, respectively; Figure 3B). The genotype effect was driven by males, which spent considerably more time emitting 22-kHz USV than their female conspecifics (Figure 3B).

During extinction on the second day, a genotype difference in the overall time spent immobile tended to emerge (G: $F_{2,87} = 2.989$, $p = 0.056$, Figure 3C). Moreover, immobility was affected by sex (S: $F_{1,87} = 10.758$, $p = 0.002$, GxS: $F_{2,87} = 2.471$, $p = 0.091$, Figure 3C), with female rats showing less immobility than males. Similarly, time spent calling during extinction was affected by genotype (G: $F_2 = 4.265$, $p = 0.017$, Figure 3D) and sex (S: $F_{1,87} = 15.040$, $p < 0.001$, Figure 3D). Furthermore, there was an interaction between genotype and sex (GxS: $F_{2,87} = 4.265$, $p = 0.017$, Figure 3D). $SERT^{-/-}$ rats spent less time calling compared to their $SERT^{+/-}$ but not $SERT^{+/+}$ littermates ($p = 0.010$ and $p = 0.157$, respectively). As during acquisition, the genotype effect was driven by males because female rats did not emit 22-kHz USV and therefore differed in time spent calling compared to their male conspecifics.

During recovery, immobility was affected by genotype (G: $F_{2,87} = 5.354$, $p = 0.007$, Figure 3E) and sex (S: $F_{1,87} = 9.115$, $p = 0.003$, GxS: $F_{2,87} = 0.066$, $p = 0.936$, Figure 3E'). $SERT^{-/-}$ rats spent more time immobile compared to their $SERT^{+/-}$ and $SERT^{+/+}$ littermates ($p = 0.015$ and $p = 0.002$, respectively). As during extinction, female rats displayed lower levels of immobility in comparison to their male conspecifics. Despite this sex difference, however, the genotype effect was robust and $SERT^{-/-}$ rats of both sexes spent more time immobile compared to their $SERT^{+/+}$ littermates ($p = 0.044$ and $p = 0.027$, respectively). Parallel to 22-kHz USV emission during acquisition and extinction, time spent emitting 22-kHz USV during recovery was affected by genotype (G: $F_{2,87} = 3.452$, $p = 0.036$, Figure 3F) and sex (S: $F_{1,87} = 6.493$, $p = 0.013$, GxS: $F_{2,87} = 1.602$, $p = 0.208$, Figure 3F). With $SERT^{-/-}$ rats lacking 22-kHz USV during recovery, time spent calling differed from their $SERT^{+/-}$ but not $SERT^{+/+}$ littermates ($p = 0.010$ and $p = 0.151$). Again, the genotype effect was driven by males because female rats spent less time calling than their male conspecifics.

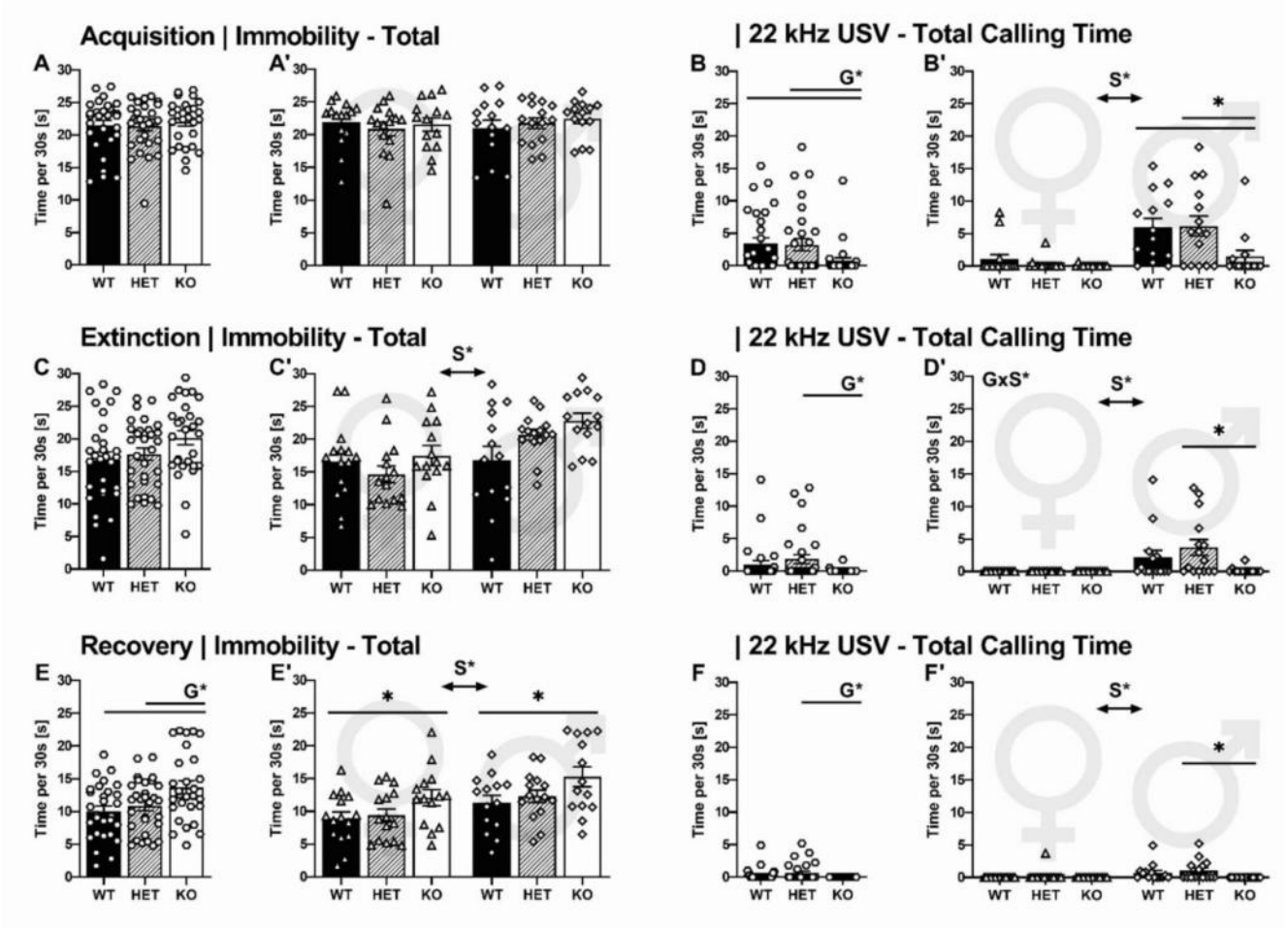

Figure 3. Overall immobility and 22-kHz total calling time. Effects of SERT deficiency on overall immobility and 22-kHz USV total calling time during acquisition (**A**,**B**), extinction (**C**,**D**), and recovery (**E**,**F**). Time spent immobile is shown for $SERT^{+/+}$ (black bar), $SERT^{+/-}$ (striped bar), and $SERT^{-/-}$ (white bar) rats with sexes pooled (**A,C,E**) and separated by sex (**A,C,E**), with females on the left and males on the right side of the panel. Time spent calling 22-kHz USV is shown for rats with sexes pooled (B,D,F) and separated by sex (**B,D,F**), with females on the left and males on the right side of the panel. N = 44 female rats (15 +/+, 15 +/−, 14 −/−), N = 43 male rats (14 +/+, 15 +/−, 14 −/−). Data are presented as mean ± SEM. G* $p < 0.05$ effect of genotype, with lines indicating significant post-hoc comparison between genotypes. S* $p < 0.05$ effect of sex. * $p < 0.05$ for subgroup comparison.

2.2.3. 22-kHz USV: Temporal Emission Pattern

The temporal 22-kHz USV emission pattern during acquisition was also affected by genotype (G: $F_{2,87} = 4.872$, $p = 0.010$) and sex (S: $F_{1,87} = 23.554$, $p < 0.001$, GxS: $F_{2,87} = 2.102$, $p = 0.129$). The number of bouts emitted by $SERT^{-/-}$ rats was lower than in $SERT^{+/-}$ and $SERT^{+/+}$ littermates ($p = 0.044$ and $p = 0.004$, respectively; Figure 4A). Similar to 22-kHz USV total calling time, the genotype effect was primarily seen in males due to the fact that female rats displayed less bouts than male rats in general (Figure 4A). When comparing bout length for vocalizing rats, however, no differences in the number of calls per bout were found between experimental conditions (G: $F_{2,87} = 0.397$, $p = 0.676$, Figure 3B; S: $F_{1,87} = 0.757$, $p = 0.392$, GxS: $F_{2,87} = 0.088$, $p = 0.916$ Figure 4B).

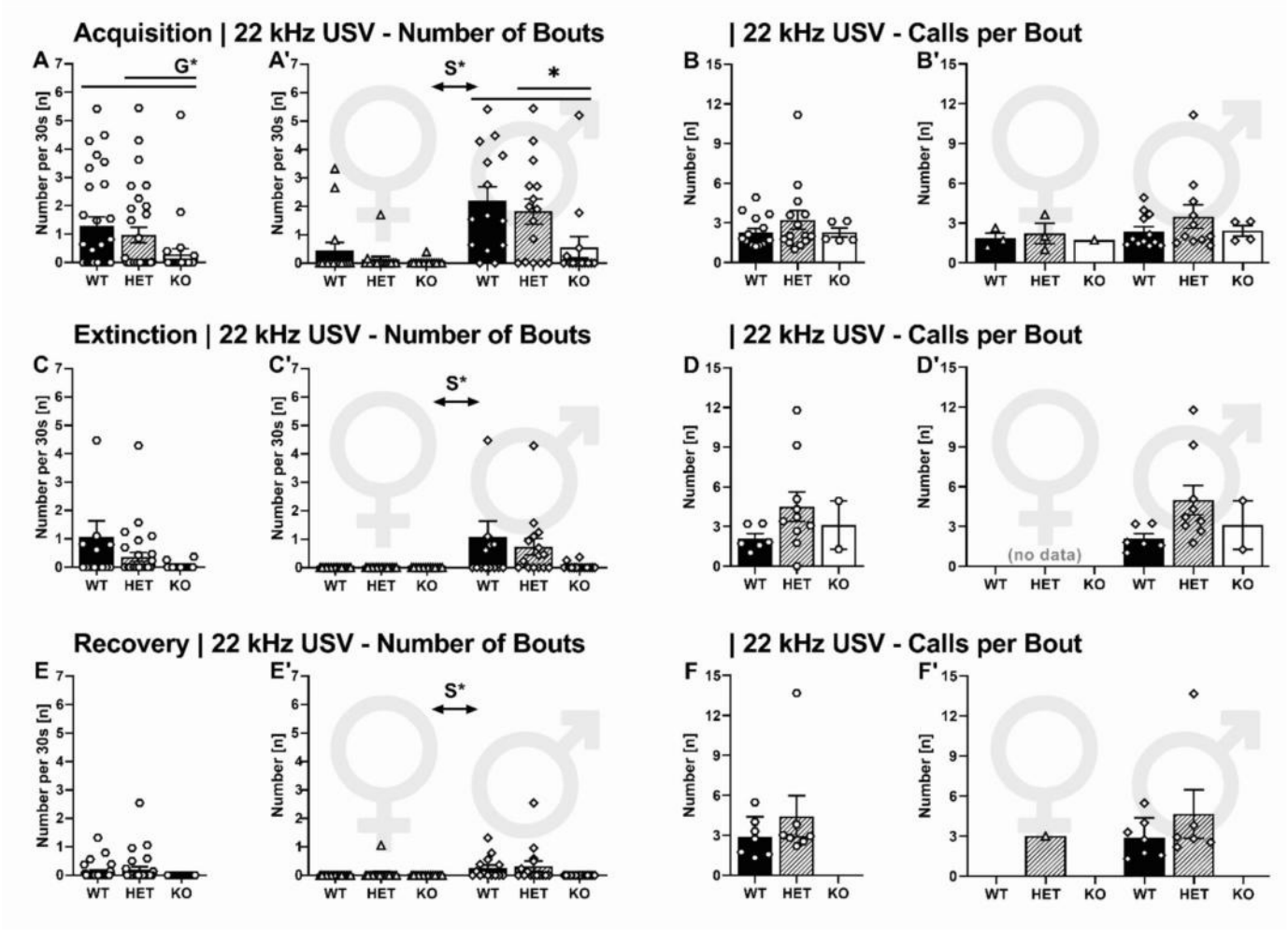

Figure 4. Temporal structure of 22-kHz USV emission. Effects of SERT deficiency on the temporal 22-kHz USV emission pattern during acquisition (**A**–**B′**), extinction (**C**–**D′**), and recovery (**E**–**F′**). The number of 22-kHz USV bouts is shown for $SERT^{+/+}$ (black bar), $SERT^{+/-}$ (striped bar), and $SERT^{-/-}$ (white bar) rats with sexes pooled (**A,C,E**) and separated by sex (**A′**,**C′**,**E′**), with females on the left and males on the right side of the panel. For rats that emitted bouts, number of calls per bout is also shown for rats with sexes pooled (**B,D,F**) and separated by sex (**B′**,**D′**,**F′**), with females on the left and males on the right side of the panel. N = 44 female (15 +/+, 15 +/− 14 −/−), N = 43 male (14 +/+, 15 +/−, 14 +/−) rats. Data are presented as mean ± SEM. G* $p < 0.05$ effect of genotype, with lines indicating significant post-hoc comparison between genotypes. S* $p < 0.05$ effect of sex. * $p < 0.05$ for subgroup comparison.

The temporal pattern of 22-kHz USV during extinction was not affected by genotype (G: F_2 = 2.066, p = 0.133, Figure 4C) but sex (S: $F_{1,87}$ = 8.777, p = 0.004, GxS: $F_{2,87}$ = 2.066, p = 0.133, Figure 4C). Due to females not displaying any 22-kHz USV during extinction, their number of 22-kHz USV bouts emitted obviously differed from their male conspecifics. Bout length did not differ between experimental conditions (G: $F_{2,87}$ = 2.199, p = 0.148, Figure 4D).

During recovery, the effects on the temporal pattern of 22-kHz USV resemble the results from the extinction phase. Due to the nearly absent 22-kHz USV from female rats, sexes differed in the number of bouts emitted (S: $F_{1,87}$ = 5.511, p = 0.021, Figure 4E′), with no effect of genotype (G: $F_{2,87}$ = 2.526, p = 0.086, GxS: $F_{2,87}$ = 1.355, p = 0.264, Figure 4E). Again, bout length did not differ between experimental conditions (G: $F_{2,87}$ = 1.003, p = 0.338, Figure 3F; S: $F_{2,87}$ = 0.226, p = 0.644, Figure 4F).

2.2.4. CS+/CS- Presentation: Immobility

To determine the effects of differential fear conditioning, immobility levels displayed during CS+ and CS- presentations were compared separately for extinction and recovery. During extinction, there was no overall difference in immobility between CS+ and CS- presentations (CS: $F_{1.000,87} = 1.643$, $p = 0.204$, Figure 4A). Furthermore, immobility levels during CS+ and CS- presentations were not affected by genotype (CSxG: $F_{2.000,87} = 2.331$, $p = 0.104$) and sex (CSxS: $F_{1.000,87} = 0.830$, $p = 0.365$, Figure 5A). Throughout the trials of CS+ and CS- presentations, immobility decreased over the time course of extinction for both CS presentations (TRIAL: $F_{4.072,87} = 49.414$, $p < 0.001$). A comparison between the first and last CS presentation revealed a difference for either type of CS (CS+: $T_{86} = 11.552$, $p < 0.001$; CS-: $T_{86} = 6.808$, $p < 0.001$, Figure 5A'). As an indication of differential conditioning, immobility decreased more rapidly for CS+ than CS- presentations (TRIALxCS: $F_{4.689,87} = 17.006$, $p < 0.001$). Particularly high immobility levels were seen during the first CS+ presentation. This was not the case for the first CS- presentation, resulting in a prominent difference in immobility levels between the first CS+ and CS- presentation ($T_{86} = 6.740$, $p < 0.001$; Figure 5A'). The pattern of immobility during extinction remained unaltered despite SERT deficiency (TRIALxG: $F_{8.144,87} = 0.700$, $p = 0.694$; TRIALxS: $F_{4.072,87} = 2.217$, $p = 0.066$; Figure 6A).

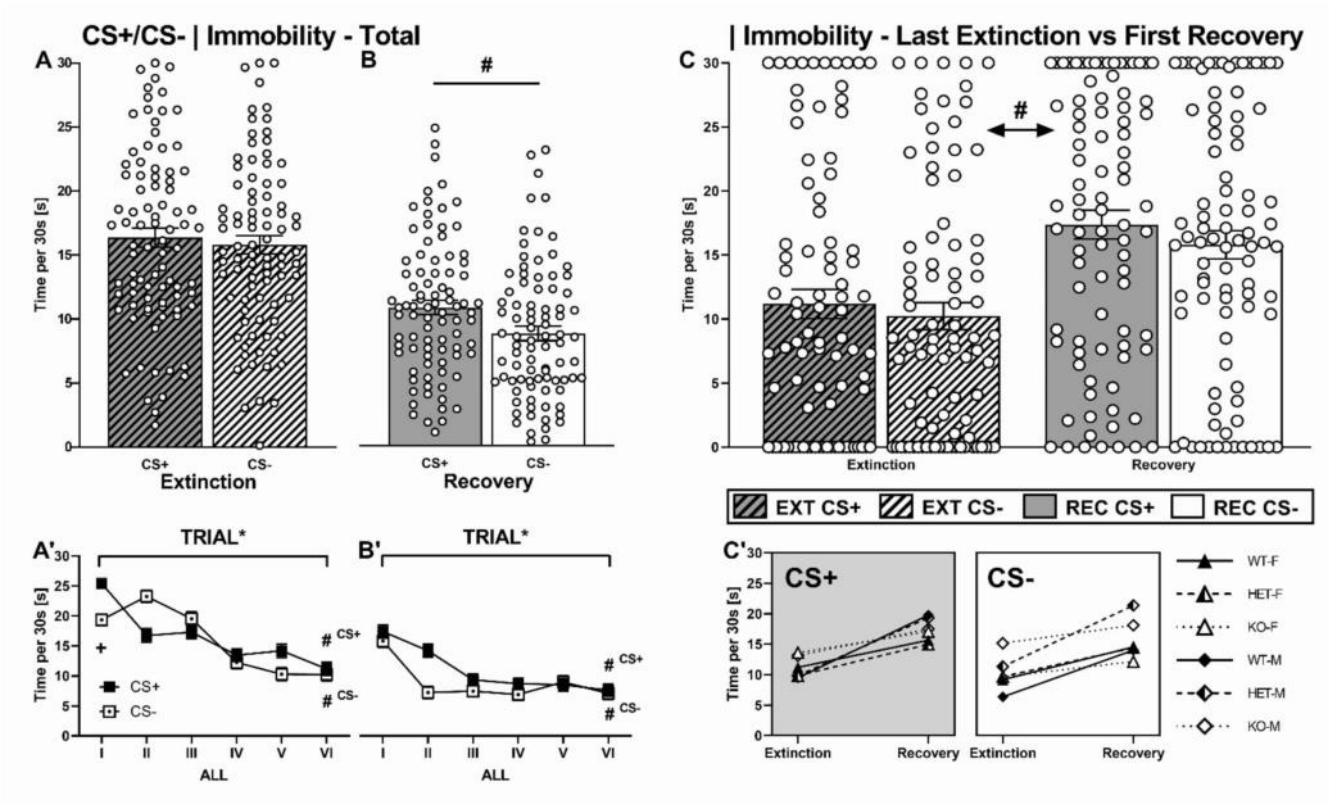

Figure 5. CS+/CS- presentation: immobility across all rats. Effects of SERT deficiency on immobility for CS+ and CS- presentations during extinction (**A**,**A'**) and recovery (**B**,**B'**), as well as the comparison of last trial extinction vs. first trial recovery (**C**–**C'**). Depicted are the total amounts of immobility for CS+ presentations (grey striped bar) and CS- presentations (white striped bar) during extinction (**A**,**C**), as well as CS+ presentation (grey bar) and CS- presentation (white bar) of recovery (**B**,**C**). Furthermore, single trial immobility levels for extinction (**A'**) and recovery (**B'**) are shown by means of CS+ presentations (black squares) and CS- presentations (white squares with dot). N = 87 rats. Data are presented as mean ± SEM. TRIAL* $p < 0.05$ effect of time course. # $p < 0.05$ significant within-subject comparison of various CS presentations. + $p < 0.05$ for within-subject comparison of first CS+ and CS- presentation. #CS+ $p < 0.05$ for within-subject comparison of first and last CS+ presentation. #CS- $p < 0.05$ for within-subject comparison of first and last CS- presentation.

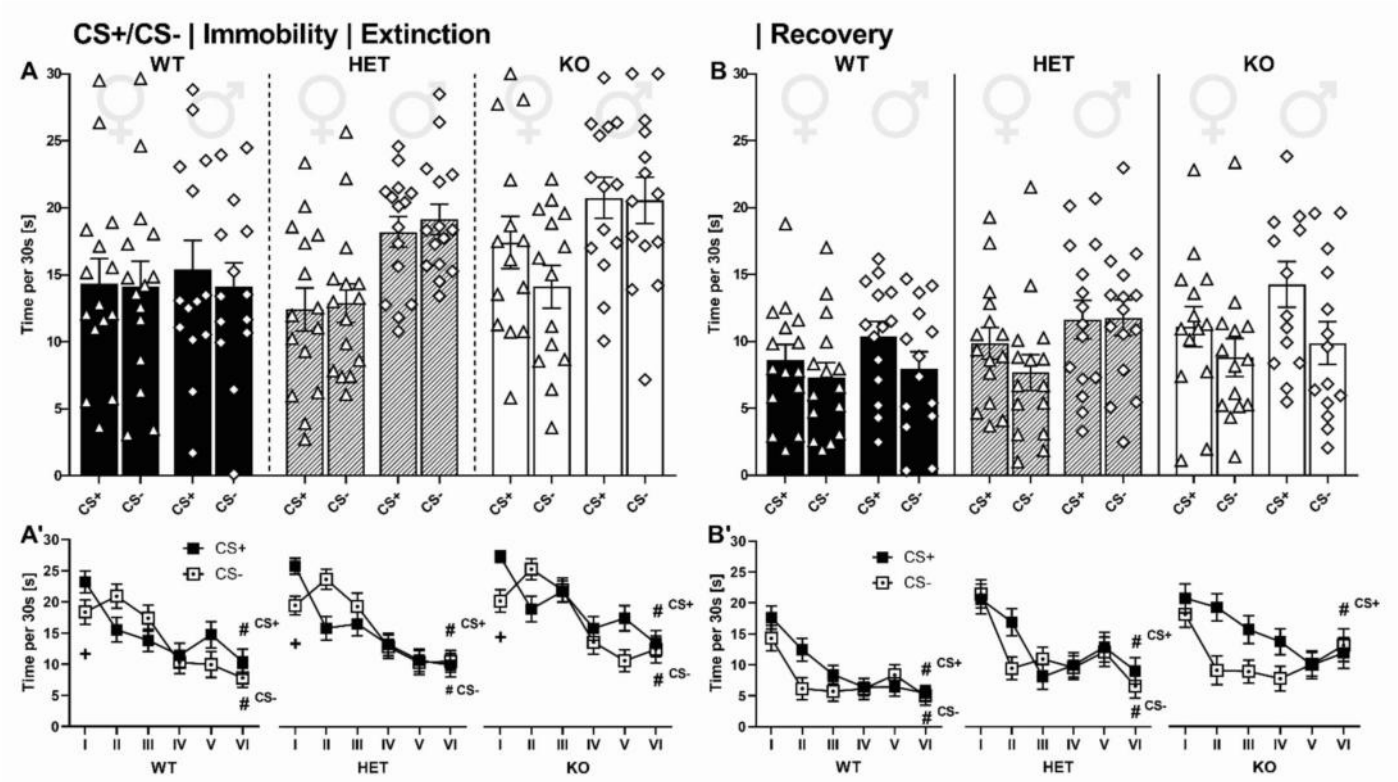

Figure 6. CS+/CS- presentation: Immobility, grouped by genotype and sex. Effects of SERT deficiency on immobility per 30 s time bin for CS+ and CS- presentation during extinction (**A**,**A'**) and recovery (**B**,**B'**). Depicted are the total amounts of immobility for both CS+ and CS- presentations for $SERT^{+/+}$ (black bar), $SERT^{+/-}$ (striped bar), and $SERT^{-/-}$ (white bar) rats. Two bars on the left comprise CS+ and CS- presentations for females; males are shown on the two right bars of every genotype. Furthermore, single trial immobility levels for extinction (**A'**) and recovery (**B'**) are shown by means of CS+ presentations (black squares) and CS- presentations (white squares with dot). N = 29 $SERT^{+/+}$ (15 female, 14 male), 30 $SERT^{+/-}$ (15 female, 15 male), 28 $SERT^{-/-}$ (14 female, 14 male) rats. Data are presented as mean ± SEM. TRIAL* $p < 0.05$ effect of time course. # $p < 0.05$ significant within-subject comparison of various CS presentations. + $p < 0.05$ for within-subject comparison of first CS+ and CS- presentation. #CS+ $p < 0.05$ for within-subject comparison of first and last CS+ presentation. #CS- $p < 0.05$ for within-subject comparison of first and last CS- presentation.

During recovery, immobility levels differed between CS+ and CS- presentations (CS: $F_{1.000,87} = 12.653$, $p = 0.001$, Figure 5B), regardless of genotype or sex (CSxG: $F_{2.000,87} = 1.336$, $p = 0.269$; CSxS: $F_{1.000,87} = 0.053$, $p = 0.818$). In general, CS+ presentations elicited more immobility than CS- presentations. Focusing on the course of immobility throughout successive trials of CS+ and CS- presentations, the response towards both CS+ and CS- presentations decreased over time (TRIAL: $F_{4.496,87} = 25.046$, $p < 0.001$), with CS- presentations showing a more rapid decline (TRIALxCS: $F_{4.262,87} = 4.253$, $p = 0.002$). Again, the amount of immobility elicited differed between the first and last trial for both stimuli (CS+: $T_{86} = 7.174$, $p < 0.001$; CS-: $T_{86} = 6.165$, $p < 0.001$, Figure 5B'). In accordance with extinction, the temporal pattern of declining immobility towards both CS+ and CS- presentations was seen across experimental conditions (TRIALxG: $F_{8.991,87} = 0.994$, $p = 0.445$; TRIALxS: $F_{4.496,87} = 0.714$, $p = 0.599$; Figure 6B').

Importantly, formerly extinguished behavior recovered. This is reflected in lower levels of immobility in response to the last CS presentation during extinction than in response to the first CS presentation during recovery a week later (EXT-REC: $F_{1.000,87} = 26.622$, $p < 0.001$, Figure 5C). Immobility during the last extinction trial was lower than during the first recovery trial for both CS+ and CS- presentations (CS: $F_{1.000,87} = 2.502$, $p = 0.118$,

Figure 5C). The recovery effect was seen irrespective of genotype and sex (all $p < 0.05$, Figure 5C, for detailed depiction of experimental conditions, see Figure S1).

2.2.5. CS+/CS- Presentation: 22-kHz USV

Similar to immobility, the overall emission of 22-kHz USV did not differ between CS+ and CS- presentations during extinction (CS: $F_{1.000,87} = 0.005$, $p = 0.942$, Figure S2A). Moreover, their emission was not modulated by genotype or sex (CSxG: $F_{2.000,87} = 0.564$, $p = 0.571$; CSxS: $F_{1.000,87} = 0.005$, $p = 0.942$, Figure S3A). However, the time spent calling 22-kHz USV varied throughout the time course of CS presentation (TRIAL: $F_{3.105,87} = 2.778$, $p = 0.040$, TRIALxCS: $F_{3.576,87} = 1.120$, $p = 0.345$ Figure S2A'). Similar to immobility, the 22-kHz USV emission differed between the first and last trial, yet only for CS+ but not CS- (CS+: $T_{86} = 2.149$, $p = 0.034$; CS-: $T_{86} = 1.000$, $p = 0.320$). This effect showed an interaction with sex (TRIALxS: $F_{3.105,87} = 2.778$, $p = 0.040$) but not genotype (TRIALxG: $F_{6.201,87} = 1.330$, $p = 0.243$). For male rats only—due to the absence of calling from their female conspecifics—22-kHz USV were emitted during early CS+ and CS- presentations, whereas no calling was detected during later time points.

In contrast to immobility, the emission of 22-kHz USV did not differ between CS+ and CS- presentations during recovery (CS: $F_{1.000,87} = 3.493$, $p = 0.065$, Figure S2B). As during extinction, their emission was not modulated by genotype or sex (CSxG: $F_{2.000,87} = 1.007$, $p = 0.370$; CSxS: $F_{1.000,87} = 1.281$, $p = 0.261$, Figure S3B). Again, throughout the time course of CS presentations, the emission of 22-kHz USV varied between sexes and genotypes – due to the virtual absence of calling from female rats and $SERT^{-/-}$ rats during recovery (TRIAL: $F_{2.038,87} = 6.475$, $p = 0.002$; TRIALxS: $F_{3.105,87} = 4.452$, $p = 0.013$, TRIALxG: $F_{4.077,87} = 2.531$, $p = 0.041$, Figure S2B'). In fact, 22-kHz USV towards CS+ presentations showed a different time course than towards CS- presentations (TRIALxCS: $F_{2.305,87} = 3.016$, $p = 0.044$, Figure S2B'), with CS+ presentations showing higher levels during the first trials than CS- presentations, albeit the decrease did not reach statistical significance at the level of CS+ and CS- presentations (CS+: $T_{86} = 0.752$, $p = 0.454$; CS-: $T_{86} = -0.295$, $p = 0.7699$).

Importantly, no evidence for recovery of 22-kHz USV emission was evident (EXT-REC: $F_{1.000,87} = 1.666$, $p = 0.685$). 22-kHz emission for both CS+ and CS- presentations did not differ (CS: $F_{1.000,87} = 1.348$, $p = 0.249$).

2.3. Additional Behavioral Assays

With the aim to identify relevant factors associated with the effects of SERT deficiency on differential fear conditioning, including acquisition, extinction, and recovery, we tested rats in additional behavioral assays, namely activity box, elevated plus maze, novel object recognition, and hot plate. These assays further allowed us to identify trait-like inter-individual differences in novelty-seeking, anxiety-related behavior, habituation learning, cognitive performance, and pain sensitivity, and to test whether such inter-individual differences predict individual performance during differential fear conditioning.

2.3.1. Activity Box

First, rats were tested in a small open field on two consecutive days to screen for novelty-seeking and habituation learning. SERT deficiency had only minor effects on horizontal and vertical locomotor activity. On both days, no differences in distance travelled were found between genotypes (G_{DAY1}: $F_{2,85} = 1.202$, $p = 0.306$; G_{DAY2}: $F_{2,84} = 1.632$, $p = 0.202$; Figure 7A) and sexes (S_{DAY1}: $F_{1,85} = 0.051$, $p = 0.822$; S_{DAY2}: $F_{1,84} = 0.743$, $p = 0.391$; GxS_{DAY1}: $F_{2,85} = 0.373$, $p = 0.690$; GxS_{DAY2}: $F_{2,84} = 1.244$, $p = 0.294$, Figure 7A'). Moreover, on day 1, no differences in rearing behavior were present (G_{DAY1}: $F_{2,85} = 1.311$, $p = 0.275$, Figure 7B; S_{DAY1}: $F_{1,85} = 2.538$, $p = 0.115$; GxS_{DAY1}: $F_{2,85} = 0.015$, $p = 0.985$, Figure 7B'). On day 2, vertical activity differed between genotypes (G_{DAY2}: $F_{2,84} = 9.263$, $p < 0.001$), with $SERT^{-/-}$ rats displaying less rearing behavior than $SERT^{+/-}$ and $SERT^{+/+}$ littermates ($p = 0.001$ and $p < 0.001$, respectively). Rearing behavior was not influenced by sex (S_{DAY2}: $F_{1,84} = 0.060$, $p = 0.807$; GxS_{DAY2}: $F_{2,84} = 326$, $p = 0.723$). Across days, distance travelled

declined, reflecting habituation learning (DAY: $F_{2.000,82} = 99.501$, $p < 0.001$). This decline was modulated by genotype (DAYxG: $F_{2.000,82} = 4.904$, $p = 0.010$) but not sex (DAYxS: $F_{1.000,82} = 0.487$, $p = 0.488$, DAYxGxS: $F_{2.000,82} = 0.277$, $p = 0.759$), with the most rapid decline in distance travelled displayed by SERT$^{-/-}$ rats compared to their SERT$^{+/-}$ and SERT$^{+/+}$ littermates ($p = 0.007$ and $p = 0.016$, respectively). Likewise, rearing behavior declined across days (DAY: $F_{1.000,82} = 115.170$, $p < 0.001$), but this decline was not modulated by genotype (DAYxG: $F_{2.000,82} = 2.335$, $p = 0.104$) or sex (DAYxS: $F_{1.000,82} = 2.735$, $p = 0.102$, DAYxGxS: $F_{2.000,82} = 0.287$, $p = 0.752$).

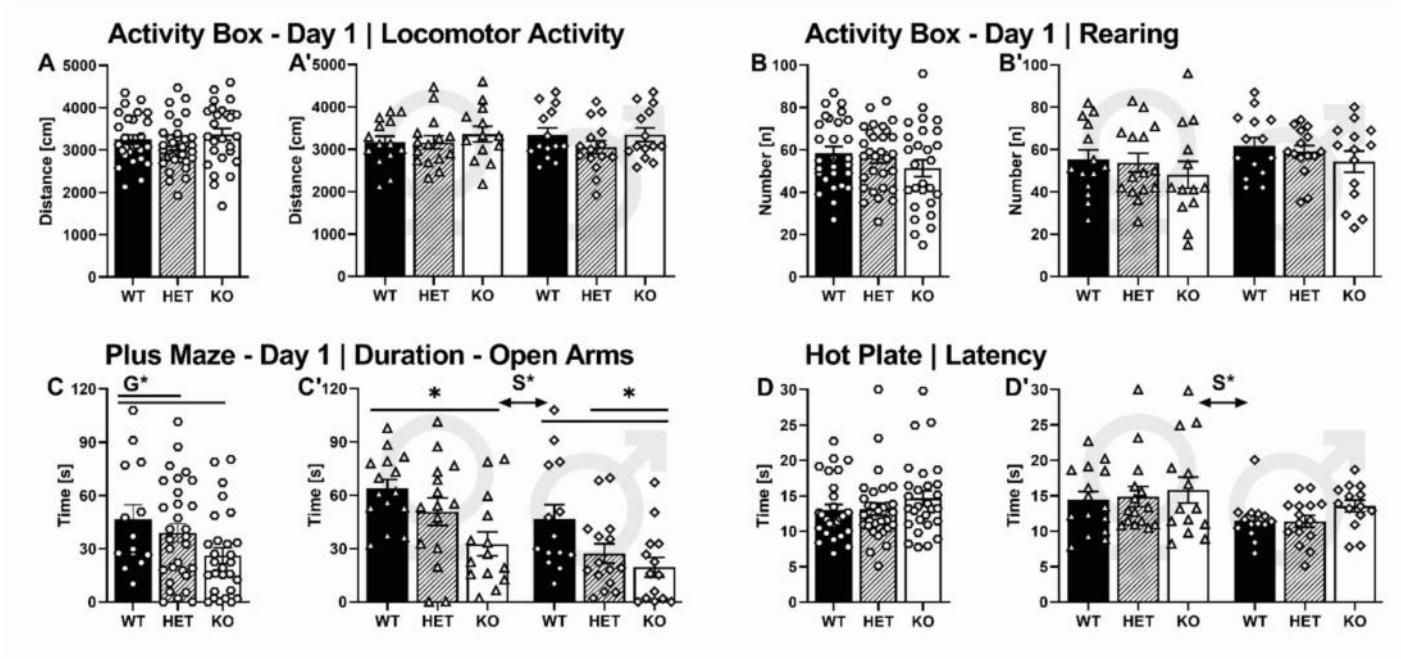

Figure 7. Additional behavioral assays. Effects of SERT deficiency on novelty-seeking in the activity box (**A**–**B′**), anxiety-like behavior in the elevated plus maze (**C**,**C′**), and pain sensitivity in the hot plate test (**D**,**D′**). Depicted are measures for SERT$^{+/+}$ (black bar), SERT$^{+/-}$ (striped bar), and SERT$^{-/-}$ (white bar) rats with sexes pooled (**A**–**D**) and separated by sex (**A′**,**B′**,**C′**,**D′**), with females on the left and males on the right side of the panel. Measures are (**A**) distance travelled in the activity box; (**B**) number of rearings in the activity box; (**C**) time spent in open arms of the elevated plus maze; and (**D**) latency to withdraw a paw in the hot plate test. N = 44 female (15 +/+, 15 +/−, 14 −/−), N = 43 male (14 +/+, 15 +/−, 14 −/−) rats, except activity box (see materials and methods for details). Data are presented as mean ± SEM. G* $p < 0.05$ effect of genotype, with lines indicating significant post-hoc comparison between genotypes. S* $p < 0.05$ effect of sex. * $p < 0.05$ for subgroup comparison.

2.3.2. Elevated Plus Maze

Next, rats were screened for anxiety-like behavior in the elevated plus maze on two consecutive days. SERT deficiency was associated with enhanced anxiety-related behavior. On the first day, open arm time differed between genotypes (G_{DAY1}: $F_{2,87} = 9.917$, $p < 0.001$, Figure 7C). SERT$^{+/+}$ rats spent more time in open arms than SERT$^{+/-}$ and SERT$^{-/-}$ littermates ($p = 0.012$ and $p < 0.001$, respectively), indicating that SERT deficiency leads to enhanced levels of anxiety-like behavior as reflected by avoidance of open spaces. Although female rats spent more time in open arms than their male conspecifics (S_{DAY1}: $F_{1,87} = 11.405$, $p = 0.001$, GxS_{DAY1}: $F_{2,87} = 0.325$, $p = 0.724$, Figure 7C′), the anxiogenic effect of SERT deficiency was robust and SERT$^{-/-}$ rats of both sexes displayed more anxiety-related behavior than their SERT$^{+/+}$ littermates ($p = 0.002$ and $p = 0.005$, respectively). On the second day, female rats still spent more time in open arms than their male conspecifics (S_{DAY2}: $F_{1,87} = 5.366$, $p = 0.023$), yet no genotype differences were detected (G_{DAY2}: $F_{2,87} = 2.266$, $p = 0.110$, GxS_{DAY2}: $F_{2,87} = 0.156$, $p = 0.856$), partly due to the reduction in

open arm time displayed by all experimental conditions irrespective of genotype and sex, reflecting intact contextual memory and the ability to adjust exploratory behavior in an anxiogenic environment (DAY: $F_{1.000,87} = 14.510$, $p < 0.001$; DAYxG: $F_{2.000,87} = 1.589$, $p = 0.210$; DAYxS: $F_{1.000,87} = 0.104$, $p = 0.709$, DAYxGxS: $F_{2.000,87} = 0.260$, $p = 0.771$). Overall locomotor activity in the elevated plus-maze on the first day was not affected by genotype (G_{DAY1}: $F_{2,87} = 2.834$, $p = 0.065$) but sex, with females displaying higher levels of locomotor activity than males (S_{DAY1}: $F_{2,87} = 6.878$, $p = 0.010$; GxS_{DAY1}: $F_{2,87} = 1.291$, $p = 0.281$). On the second day, no differences between genotypes and sexes were apparent (G_{DAY2}: $F_{2,87} = 0.467$, $p = 0.629$; S_{DAY2}: $F_{2,87} = 2.679$, $p = 0.106$; GxS_{DAY2}: $F_{2,87} = 0.453$, $p = 0.638$). Locomotor activity declined across days (DAY: $F_{1.000,87} = 77.413$, $p < 0.001$), irrespective of genotype (DAYxG: $F_{2.000,87} = 0.665$, $p = 0.417$) and sex (DAYxS: $F_{1.000,87} = 0.806$, $p = 0.450$, DAYxGxS: $F_{2.000,87} = 0.312$, $p = 0.733$).

2.3.3. Novel Object Recognition

Cognitive performance in the novel object recognition test was not affected by SERT deficiency. The ability to recognize familiar objects and to differentiate them from novel objects was tested in the novel object recognition test after a delay of 30 min. When given the opportunity to explore a novel object simultaneously with a familiar object, rats preferred the novel object independent of genotype and sex (OBJ: $F_{1.000,87} = 54.416$, $p < 0.001$, OBJxG: $F_{2.000,87} = 0.257$, $p = 0.774$, OBJxS: $F_{1.000,87} = 1.183$, $p = 0.280$, OBJxGxS: $F_{1.000,87} = 0.086$, $p = 0.918$, Figure S4), as reflected in novel object investigation times above chance level in all experimental conditions (all $p < 0.05$). Of note, object exploration displayed during the acquisition phase did not differ between genotypes (G: $F_{2.000,87} = 1.960$, $p = 0.148$) but sex, with males exploring more than females (S: $F_{1.000,87} = 19.724$, $p < 0.001$, GxS: $F_{2.000,87} = 0.249$, $p = 0.781$).

2.3.4. Hot Plate

Effects of SERT deficiency on pain reactivity to thermal stimulation were not found. The latencies to withdraw the paws from a hot surface were comparable between genotypes (G: $F_{2,87} = 1.200$, $p = 0.307$, Figure 7D). However, female rats displayed higher withdrawal latencies compared to their male conspecifics (S: $F_{1,87} = 8.488$, $p = 0.005$, GxS: $F_{2,87} = 0.131$, $p = 0.877$, Figure 7D'), indicating less pain reactivity to thermal stimulation in females.

2.4. Predictors of Inter-Individual Differences in Immobility

Overall, rats spending a lot of time immobile during the acquisition phase were also prone to show higher levels of immobility during subsequent phases of the differential fear conditioning paradigm, namely extinction and recovery (Figure 8). For one, immobility displayed in response to tone-shock pairings during acquisition (ACQ) was positively associated with immobility during extinction (ACQ-EXT: $r = 0.423$, $p < 0.001$, Figure 9A) and recovery (ACQ-REC: $r = 0.240$, $p = 0.025$, Figure 9B). Even more so, immobility levels in the extinction context reliably predicted immobility levels during recovery in the same context seven days later (EXT-REC: $r = 0.656$, $p < 0.001$, Figure 9C). This indicates that variability in immobility was in part due to stable inter-individual differences that were reliably identified throughout the different phases of the differential fear conditioning paradigm.

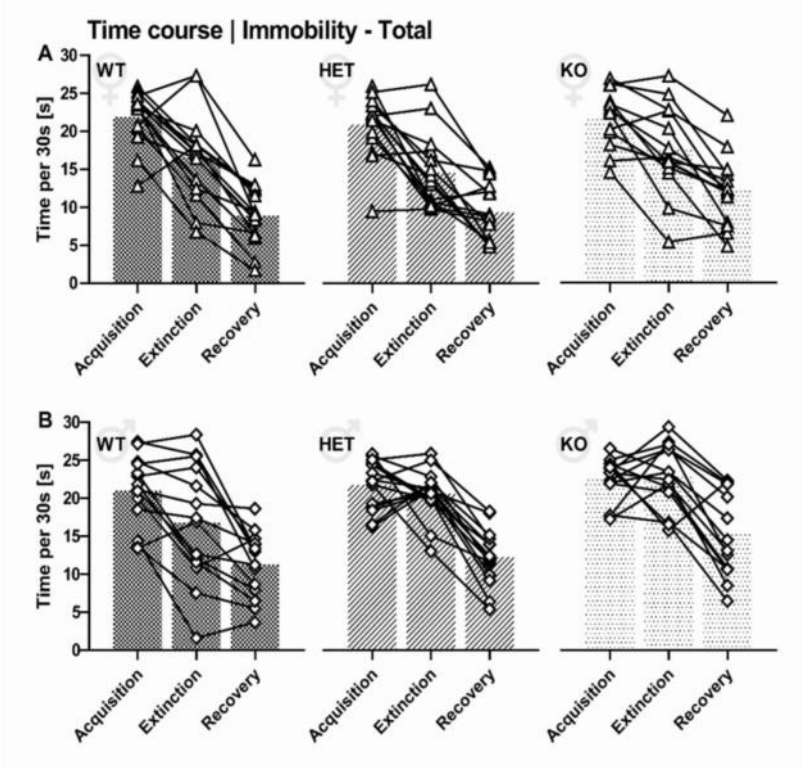

Figure 8. Inter-individual differences in immobility. Effects of SERT deficiency on immobility during acquisition, extinction, and recovery, depicted as individual values for female (**A**, with triangles) and male (**B**, white squares) rats. Additionally, means for $SERT^{+/+}$ (checkered bars), $SERT^{+/-}$ (striped bars), and $SERT^{-/-}$ (dotted bars) rats are shown. N = 44 female (15 +/+, 15 +/+, 14 −/−), N = 43 male rats (14 +/+, 15 +/+, 14 −/−) rats.

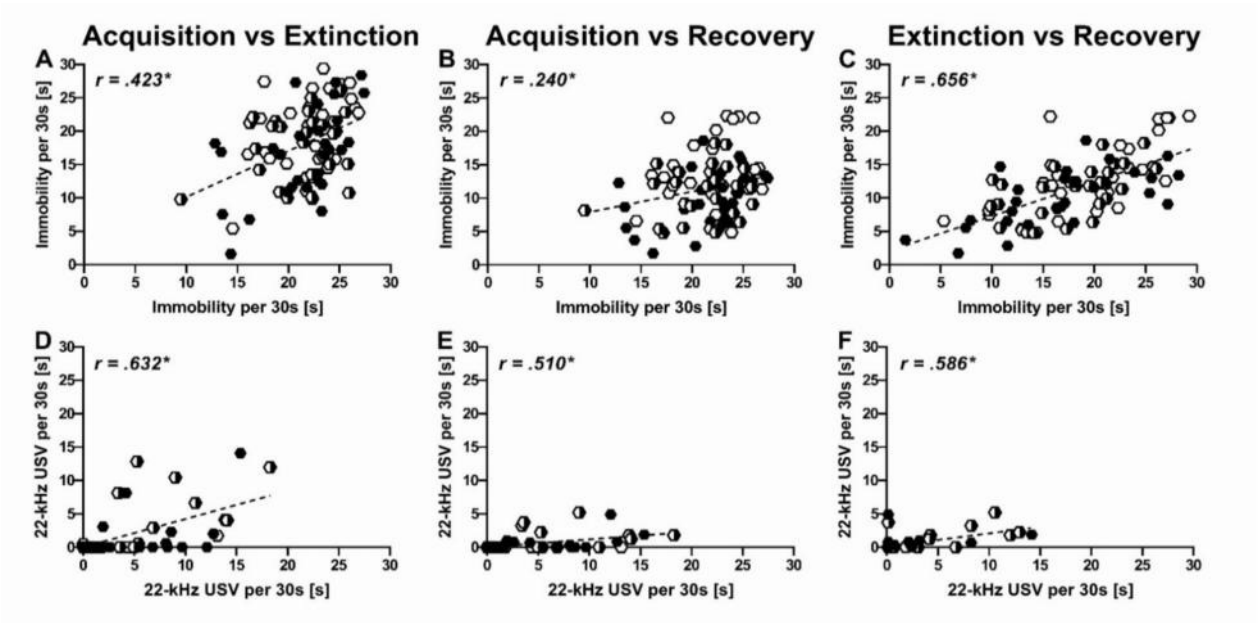

Figure 9. Correlation between immobility and 22-kHz USV emission. Duration of immobility (**A–C**) and 22-kHz USV (**D–F**) during acquisition in relation to extinction (**A,D**) and recovery (**B,E**), and during extinction and recovery (**C,F**), respectively, with individual values for $SERT^{+/+}$ (black diamond), $SERT^{+/-}$ (black-white diamond), and $SERT^{-/-}$ (white diamond) rats. N = 29 $SERT^{+/+}$ (15 female, 14 male), 30 $SERT^{+/-}$ (15 female, 15 male), 28 $SERT^{-/-}$ (14 female, 14 male) rats. Statistical significance ($p < 0.05$) of correlation coefficients is indicated in bold and italic.

Similar to immobility, there were stable inter-individual differences in the emission of 22-kHz USV. Specifically, 22-kHz USV emission during acquisition was positively associated with 22-kHz USV emission during extinction (ACQ-EXT: r = 0.632, $p < 0.001$, Figure 9D) and recovery (ACQ-REC: r = 0.510, $p < 0.001$, Figure 9E). Moreover, 22-kHz USV emission during extinction was positively associated with 22-kHz USV emission during recovery (EXT-REC: r = 0.586, $p < 0.001$, Figure 9F). Unexpectedly, however, the association between 22-kHz USV emission and immobility was weak. While there was a positive correlation during acquisition (ACQ-ACQ: r = 0.259, $p < 0.015$, Figure S5A), this was not the case during extinction (EXT-EXT: r = 0.130, $p = 0.230$, Figure S5B) and recovery (REC-REC: r = 0.087, $p = 0.425$, Figure S5C). Moreover, 22-kHz USV emission in response to tone-shock pairings during acquisition did not predict immobility levels during extinction (ACQ-EXT: r = 0.116, $p = 0.284$) and recovery (ACQ-REC: r = 0.028, $p = 0.800$). This suggests that 22-kHz USV emission and immobility are at least partially distinct components of the fear response. Of note, body weight was correlated with inter-individual differences in immobility in females during extinction but not acquisition or recovery (BW-ACQ: r = 0.055, $p = 0.724$; AB-EXT: r = 0.405, $p = 0.007$; AB-REC: r = 0.231, $p = 0.136$). No prominent association between body weight and immobility was obtained in males (BW-ACQ: r = −0.091, $p = 0.566$; AB-EXT: r = −0.156, $p = 0.324$; AB-REC: r = −0.041, $p = 0.797$). Body weight did not correlate with the emission of 22-kHz USV in males and females (all $p > 0.05$).

Because recovery was reflected in immobility levels but not 22-kHz USV emission, we thus asked whether immobility during differential fear conditioning can be predicted by trait-like inter-individual differences in novelty seeking, anxiety-related behavior, habituation learning, cognitive performance, and pain sensitivity. Locomotor activity during the first exposure to the activity box (AB) did not predict immobility levels displayed during acquisition, extinction, or recovery (AB-ACQ: r = 0.102, $p = 0.355$; AB-EXT: r = −0.057, $p = 0.603$; AB-REC: r = −0.101, $p = 0.358$, Figure 10A–A″). Likewise, habituation learning displayed in response to the repeated exposure to the activity box did not predict immobility levels (AB-ACQ: r = −0.093, $p = 0.405$; AB-EXT: r = −0.118, $p = 0.287$; AB-REC: r = −0.115, $p = 0.302$. Avoidance of the open arms in the elevated plus maze (EPM), however, was associated with higher levels of immobility during extinction and recovery (EPM-EXT: r = 0.423, $p < 0.001$, Figure 10B′; EPM-REC: r = 0.240, $p = 0.025$, Figure 10B″) but not in the acutely threating situation of tone-shock pairings during acquisition (EPM-ACQ: r = 0.015, $p = 0.893$, Figure 10B). The typically seen reduction in open arm time from the first to the second exposure to the elevated plus maze was not associated with immobility levels displayed during acquisition, extinction, or recovery (EPM-ACQ: r = 0.075, $p = 0.490$; EPM-EXT: r = 0.016, $p = 0.883$; EPM-REC: r = 0.065, $p = 0.551$). Finally, immobility during different phases of differential fear conditioning was neither predicted by cognitive performance in the novel object recognition test (OBJ-ACQ: r = −0.086, $p = 0.431$; OBJ-EXT: r = 0.016, $p = 0.882$; OBJ-REC: r = 0.017, $p = 0.877$) nor pain sensitivity in the hot plat test (HP-ACQ: r = 0.059, $p = 0.589$; HP-EXT: r = −0.075, $p = 0.489$; HP-REC: r = −0.038, $p = 0.725$).

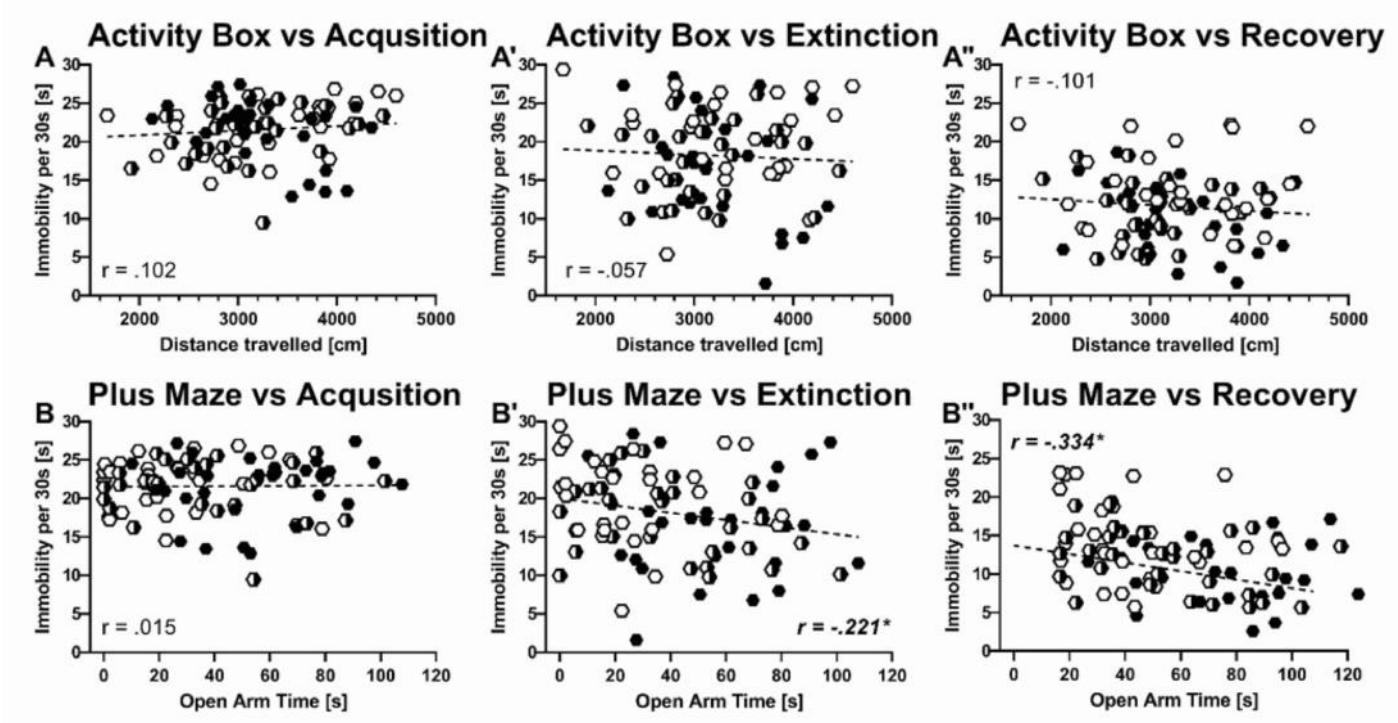

Figure 10. Correlation: novelty-seeking, anxiety-related behavior, and immobility. Duration of immobility during acquisition, extinction, and recovery, in relation to novelty-seeking in the activity box (**A**–**A″**) and anxiety-like behavior in the elevated plus maze (**B**–**B″**). Depicted are $SERT^{+/+}$ (black diamond), $SERT^{+/-}$ (black-white diamond), and $SERT^{-/-}$ (white diamond) rats. N = 29 $SERT^{+/+}$ (15 female, 14 male), 30 $SERT^{+/-}$ (15 female, 15 male), 28 $SERT^{-/-}$ (14 female, 14 male) rats, except activity box (see materials and methods for details). Data are presented as individual values and correlation coefficients. Statistical significance ($p < 0.05$) of correlation coefficient is indicated in bold and italic.

3. Discussion

In the present study, we aimed at identifying key factors associated with inter-individual differences in fear extinction in rats and assessed fear-related behavior through quantifying the emission of alarm 22-kHz USV in addition to the commonly applied measure immobility. We found that SERT deficiency strongly affected the emission of 22-kHz USV during differential fear conditioning. During acquisition, extinction, and recovery, SERT deficiency consistently led to a reduction in 22-kHz USV emission. In line with our previous report [37], most 22-kHz USV were emitted during acquisition when repeated tone-shock pairings were presented, whereas calling behavior declined in subsequent phases or was abolished completely depending on genotype and sex. Specifically, besides their already reduced 22-kHz USV emission rates during acquisition, rats lacking SERT but not wildtype littermate controls virtually ceased to emit 22-kHz USV during extinction and subsequent recovery. This was seen in male and female rats, albeit 22-kHz USV emission was comparatively low in females. A different pattern was evident for immobility. SERT deficiency did not affect immobility during acquisition. Genotype differences started to emerge during extinction, however, and during recovery rats lacking SERT showed much higher levels of immobility than wildtype littermate controls. With the aim to identify relevant factors associated with the effects of SERT deficiency on differential fear conditioning, we tested rats in additional behavioral assays, namely activity box, elevated plus maze, novel object recognition, and hot plate. Rats lacking SERT behaved similar to their littermates in those assays, with the expectation of the elevated plus maze, where they engaged in considerably higher levels of anxiety-related behavior. Finally, we studied predictors of inter-individual differences in immobility during differential fear conditioning and found that immobility displayed in response to tone-shock pairings during acquisition

predicted immobility levels during extinction and recovery. The predictive quality of 22-kHz USV was low. Among the trait-like inter-individual differences in novelty seeking, anxiety-related behavior, habituation learning, cognitive performance, and pain sensitivity, anxiety-related behavior had the best predictive quality.

3.1. Fear-Related Behavior

Tone-shock pairings during the acquisition phase of the differential fear conditioning paradigm led to high levels of immobility, followed by a gradual decline throughout phases of extinction and recovery. During extinction, immobility towards the tone that was previously accompanied by an electric stimulation (CS+) was stronger at the outset and decreased more rapidly in the course of training, compared to the stimulus that was never paired with foot shocks (CS-). Similarly, during recovery, rats discriminated between CS+ and CS- presentations and displayed higher immobility levels during CS+ presentations. For both CS presentations, extinguished behavior recovered, as reflected in the lower levels of immobility in response to the last CS presentation during extinction than in response to the first CS presentation during recovery a week later. Thus, our results indicate that differential conditioning was accompanied by substantial conditioning towards the CS- that was never paired with foot shocks. Various possibilities for substantial conditioning towards the CS- are conceivable. Due to similarities between CS+ and CS-, fear responses induced by the CS+ might have been generalized to the CS- [56]. Moreover, the context might have contributed to the responding towards the CS-. Fear-related responses can be induced by the context itself and a clear discrimination of context and cue in fear conditioning paradigms is difficult, where the context can be interpreted as combination of different cues [56]. Even though contextual cues, most notably odor and visual patterning, differed between acquisition and extinction, there might have been some degree of generalization between the contexts, which may have elevated responding towards the CS-.

Consistent with our previous studies, the majority of 22-kHz USV was emitted during acquisition [37,52–59]. Much lower 22-USV emission rates were seen during extinction and recovery. No recovery effect was evident for 22-kHz USV emission. In contrast to immobility, the emission of 22-kHz USV did not differ between CS+ and CS- presentations during extinction, whereas during recovery, there was evidence for differential 22-kHz USV emission towards CS+ and CS-.

3.1.1. Sex Differences in Fear-Related Behavior

Male and female rats showed equal levels of immobility during acquisition. During extinction and recovery, however, female rats displayed lower levels of immobility. Consistent sex differences were seen in the emission of 22-kHz USV during acquisition, extinction, and recovery of differential fear conditioning. During acquisition, female rats showed a lower prevalence of 16% to emit 22-kHz USV compared to their male conspecifics with 63%, similar to our previous report [37]. During extinction and recovery, female rats virtually ceased to emit 22-kHz USV with the exception of one vocalizing female during recovery as compared to 40% of males during extinction and still 30% during recovery. The low prevalence of calling in female rats is reflected in less time spent calling and fewer numbers of bouts emitted as well. Interestingly, vocalizing females showed no difference in the temporal 22-kHz USV emission pattern and bout length did not differ between males and females in contrast to previous findings [37]. This inconsistency might be due to the fact that a differential fear conditioning paradigm was applied here. Even though the number of tone-shock pairings was the same in the present and the previous study, the addition of CS- presentations might have affected the temporal 22-kHz USV emission pattern. For instance, the additional tone presentations could have interfered with emitting 22-kHz USV in bouts.

The mechanism underlying sex differences in 22-kHz USV emission remains unclear. For one, similar sex differences were seen in other aversive experimental settings, with

female rats emitting fewer 22-kHz USV when confronted with air puffs [45,46] or electric shocks [60–62]. Interestingly, there is a report on reduced 22-kHz USV emission in female rats despite higher foot shock sensitivity, where relatively low shock levels were found to be sufficient to induce sonic squeaks associated with pain in female but not male rats [63]. Therefore, dose-response curves for 22-kHz USV induced by foot shock application might differ between male and female rats. For male rats, a positive relation between foot shock intensity and 22-kHz USV was reported, with foot shocks of 0.5 mA being sufficient to induce 22-kHz USV in the majority of rats [57]. For female rats, dose-response studies on 22-kHz USV evoked by foot shocks are still to be conducted and it appears possible that the higher sensitivity to foot shocks in females might result in greater perceived imminence of threat, which in turn can lead to the immediate cessation of 22-kHz USV emission [64]. In the present study, however, female rats displayed lower pain sensitivity in the hot plate test performed shortly after the differential fear conditioning, complicating the relation of 22-kHz USV, sensitivity to foot shock, and perception of pain.

On the other hand, opposing sex differences in 22-kHz USV emission were seen under more naturalistic conditions. In response to a predator, female rats living in social groups in a visible burrow system emit more 22-kHz USV than their male conspecifics [65,66]. Other than solely expressing anxiety and fear, 22-kHz USV are thought to function as alarm calls to warn conspecifics about threats and were shown to evoke a fear response in receiver rats [42,65]. The latter was also observed during social fear conditioning [59,67,68] and confirmed in playback studies, where 22-kHz USV induced behavioral inhibition in receiver rats [43,69]. Supporting a communicative function, 22-kHz USV emission was reported to be potentiated by the presence of conspecifics [42] and it appears possible that this audience effect is more prominent in female than male rats.

Finally, the direction of sex differences in 22-kHz USV evoked by foot shock may be heavily influenced by strain. Male Sprague-Dawley rats were reported to produce more 22-kHz USV than their female conspecifics, whereas the opposite was shown for Long-Evans rats [61]. Interestingly, the studies conducted in naturalistic environments used Long-Evans rats, whereas sex differences in 22-kHz USV induced by foot shock were obtained in Sprague-Dawley rats [63], and 22-kHz USV elicited by air puffs in outbred Wistar and inbred F344 rats [46]. A systematic study on sex differences in the emission of 22-kHz USV including multiple elicitors of 22-kHz USV appears therefore to be warranted.

3.1.2. Genotype Differences in Fear-Related Behavior

Effects of SERT deficiency did not affect immobility during the acquisition phase of the differential fear conditioning paradigm and no differences were seen between $SERT^{+/+}$, $SERT^{+/-}$, and $SERT^{-/-}$ rats. This is in line with previous studies and suggests intact acquisition of conditioned fear despite SERT deficiency [30,32,33,37]. During extinction on the next day, however, effects of SERT deficiency started to emerge and prominent genotype differences were evident during the recovery phase a week later. Here, $SERT^{-/-}$ rats, especially $SERT^{-/-}$ males, displayed higher levels of immobility compared to $SERT^{+/+}$ and $SERT^{+/-}$ rats. This supports previous findings obtained in rats lacking SERT [28], which displayed a reduced ability to extinguish fear-related behavior in several studies [23,25,29–35], with some of them suggesting that the lack of SERT also impedes extinction recall [32,34]. In our own previous study, however, we did not see an effect of SERT deficiency on extinction [37]. The fact that genotype differences were evident in the present study might be related to the presentation of a CS- in addition to a CS+. The CS- presentation might have helped to reveal genotype effects due to a weakening of ceiling effects of otherwise high baseline immobility levels elicited by tone-shock pairings.

Similar to sex, SERT deficiency also strongly affected the emission of 22-kHz USV during differential fear conditioning. This is consistent with our previous report [37]. During acquisition, only 18% of $SERT^{-/-}$ rats emitted 22-kHz USV compared to 52% of $SERT^{+/+}$ and 47% of $SERT^{+/-}$ rats. Similar patterns were obtained during extinction and recovery, although at a much lower level. In line with the significantly lower prevalence in 22-kHz

USV of SERT$^{-/-}$ rats, the total calling time of SERT deficient rats was significantly reduced in all phases of differential fear conditioning. Additionally, SERT$^{-/-}$ male rats emitted less bouts during acquisition. Yet again, bout length did not differ between genotypes.

There are many neurobiological differences potentially contributing to these genotype effects. One of them is alterations in the function of the amygdala. The amygdala is known to play a key role in the acquisition of fear [70] and the production of 22-kHz USV was found to be orchestrated by an interplay of several nuclei of the amygdala [71]. For example, the medial nucleus [72] as well as the basolateral nucleus of the amygdala [73] were reported to mediate 22-kHz USV during conditioned avoidance behavior. However, the central nucleus of the amygdala is widely believed to exert the most dramatic effects on 22-kHz USV production [71]. In fact, removal [74] or neurotoxic lesion [75] of the central nucleus of the amygdala blocked 22-kHz USV as a fear-related conditional response. Interestingly, SERT$^{-/-}$ rats exhibit diverging neuronal activity in the central nucleus of the amygdala [33] and this might contribute to changes in 22-kHz USV production. Other factors potentially contributing to the genotype differences in fear-related behavior are alterations in 5-HT receptor expression or sensitivity. In fact, SERT deficiency was reported to be associated with changes in 5-HT receptors. In SERT$^{-/-}$ rats, the 5-HT1A receptor is desensitized [76–78] and 5-HT3 receptor function is altered [79]. In SERT$^{-/-}$ mice, 5-HT1A and 5-HT1B autoreceptor binding and function is altered [80,81], as well as 5-HT2A/2C receptor density [82,83]. These changes are observed in a region-specific manner. Such receptor changes could have contributed to the changes in immobility and 22-kHz USV emission. Future studies employing (local) genetic or pharmacological manipulations will be needed for further understanding.

3.2. Relation of 22-kHz USV and Immobility

While sex differences in immobility and 22-kHz USV emission point in the same direction, with male rats spending more time immobile and vocalizing more than female rats, genotype differences are inconsistent. Despite the fact that rats lacking SERT display more immobility, they do not emit more 22-kHz USV than littermate controls. A dissociation of immobility and 22-kHz USV emission has been previously reported [37,45]. Although immobility and 22-kHz USV emission are both thought to reflect enhanced anxiety and fear, this suggests that they reflect at least in part different aspects of the fear response. In rats lacking SERT, high levels of immobility were found to be associated with exaggerated threat-related bradycardia and related findings were obtained in human carriers of the short 5-HTTLPR allelic variant [35]. Because heart rate was linked to 22-kHz USV emission, this could explain why 22-kHz USV emission is reduced in rats lacking SERT. In fact, pharmacological studies targeting β-adrenergic receptors suggest that the emission of 22-kHz USV is positively associated with heart rate [84]. Moreover, acoustic features, such as small changes in peak frequency, were reported to be correlated with blood pressure and heart rate [85].

The view that immobility and 22-kHz USV emission reflect at least in part different aspects of the fear response is supported by the fact that they were positively correlated during the acquisition phase but not during extinction and recovery. Moreover, 22-kHz USV emission during tone-shock pairings did not predict immobility levels during subsequent phases, suggesting a low predictive quality of the acute 22-kHz USV response for conditioned fear. This dissociation might be due to only partially overlapping neuronal circuits controlling both components of the fear response. In fact, diverging pathways from the basolateral nucleus of the amygdala and the central nucleus of the amygdala were associated with immobility and 22-kHz USV emission, respectively. It was shown that neurotoxic lesions of the basolateral nucleus of the amygdala impair both immobility and 22-kHz USV, whereas neurotoxic lesions of the central nucleus of the amygdala impair the emission of 22-kHz USV in a greater fashion [75].

Together, this indicates that the emission of 22-kHz USV is associated with a negative affective state but that their emission is not a simple reflection of a negative affective

state. A sufficiently strong negative affective state appears to be a necessary condition for 22-kHz USV to occur. However, whether a negative affective state indeed leads to 22-kHz USV appears to be dependent on other factors, such as sex, genotype, and social factors, including the presence of conspecifics [42]. This is most likely due to the communicative function of 22-kHz USV as alarm calls [42,65]. Immobility, in contrast, has different functions and is considered to occur in an attentive action preparation phase during threat exposure. While 22-kHz USV emission most likely increases the risk of being detected by a predator, immobility is supposed to reduce the likelihood of being detected. A careful differentiation between affective state and the expression of the affective state appears warranted.

3.3. Trait-Like Inter-Individual Differences

Sexes did not differ in novelty-seeking and habituation learning in the activity box and cognitive performance during novel object recognition was similar in male and female rats. In the elevated plus maze, however, male rats displayed more anxiety-related behavior than female rats. Moreover, pain sensitivity was higher in male than female rats. Both sex differences might be associated with the differences between male and female rats seen during differential fear conditioning. Heightened anxiety and pain sensitivity in male rats might have contributed to the higher levels of immobility during extinction and recovery and the increased level of 22-kHz USV emission throughout all three phases of differential fear conditioning, albeit elevated levels of 22-kHz USV in male rats was evident in our previous report in absence of differences in pain sensitivity [37].

SERT deficiency had no major effects on novelty-seeking in the activity box, cognitive performance assessed during novel object recognition, and pain sensitivity quantified in the hot plate test. This is consistent with previous reports [22,37,86]. Of note, novel recognition deficits were previously reported in studies applying longer inter-trial intervals of more than 30 min [86,87]. Therefore, genotype differences in 22-kHz USV emission and immobility displayed during differential fear conditioning do not appear to be due to unspecific effects associated with altered levels of exploratory behavior, such as very low or very high activity levels. Moreover, severe cognitive impairments can be excluded as the driving force. Likewise, differences in pain sensitivity do not appear to play a major role. Finally, the fear extinction deficit displayed by rats lacking SERT does not appear to be due to a general deficit in habituation learning. In fact, rats lacking SERT displayed the most robust decline in locomotor activity when exposed to the activity box a second time.

However, SERT deficiency affected anxiety-related behavior in the elevated plus maze. In both sexes, a clear gene dosage effect was evident and anxiety-related behavior was robustly increased in rats lacking SERT. Increased anxiety-related behavior was consistently seen in previous studies applying the elevated plus maze [22–26,88] as well as other paradigms suitable to reveal effects on anxiety, such as light-dark test [22–25,88] and novelty suppressed feeding [25]. This suggests that the alterations displayed by rats lacking SERT during differential fear conditioning might at least partly be driven by higher trait anxiety.

However, this does not appear to be the case for 22-kHz USV emission. The increase in anxiety-related behavior appears to be in contrast to the reduction in 22-kHz USV emission displayed by rats lacking SERT. In previous studies, 22-kHz USV emission was linked to high trait anxiety, and it was shown that rats that displayed high levels of anxiety-related behavior in the elevated plus maze emitted particularly high numbers of 22-kHz USV when challenged with tone-shock pairings during fear conditioning [53]. Therefore, one would have expected higher levels of 22-kHz USV emission in rats lacking SERT and not lower 22-kHz USV emission rates. In contrast, the increase in anxiety-related behavior displayed by rats lacking SERT might underlie the higher level of immobility during the recovery phase of the differential fear conditioning paradigm. In fact, trait anxiety was found to predict enhanced fear memory after fear conditioning in mice [89] and rats [68]. Moreover, rats selectively bred for high anxiety display deficits in extinction and extinction

recall [90]. Related to that, inter-individual differences in extinction were found to result in systematic variation in recovery, where slow extinguishing rats were more prone to the relapse of fear than their fast-extinguishing conspecifics [91]. Finally, there is also evidence that anxious rats display higher levels of immobility [53,92], enhanced discrimination of fear-relevant cues [93], but slower active avoidance learning [94] associated with alterations in 5-HT concentrations [95].

Finally, SERT deficiency also affected body weight gain and body weight was reduced in rats lacking SERT. This effect was most prominent in males. Reports on the effects of SERT deficiency on body weight are rare, with a few exceptions. For example, one study found a reduction only during early life [87] and another one only in females [21] but not in males, as in the present study. However, it appears unlikely that body weight had prominent effects. Correlations between body weight and immobility or 22-kHz USV emission were rarely observed. While it is difficult to see how the genotype difference in body weight might directly contribute to the behavioral differences, body weight is often used as a proxy for the rank in a social hierarchy and the dominance structure was shown to be associated with the emission of 22-kHz USV [96]. Moreover, it was reported that social dominance status in rats predicts social fear transmission in rats. Following a social interaction with a fear conditioned dominant rat, subordinate rats displayed enhanced fear responses, possibly driven by the emission of 22-kHz USV [97]. It thus would be interesting to test whether SERT deficiency affects the social hierarchy.

3.4. Clinical Implications

In humans, the 5-HT system including the 5-HTTLPR plays a key role in the etiology of anxiety disorders and affects treatment efficacy. Specifically, the short allelic variant associated with reduced transcription and altered function of SERT leads to an increased risk of developing PTSD after high trauma exposure [19] and reduces treatment efficacy of exposure-based therapy [98]. While immediate results of exposure-based therapy were found to be indistinguishable in long and short allele carriers [99], short but not long allele carriers displayed strong return of fear [98,99], similar to SERT deficient rats in the present study. Because amygdala activation was repeatedly associated with differences in fear extinction across species [18,25,33,100], it would be interesting to see whether targeted amygdala manipulations might help to improve fear extinction in SERT deficient rats.

4. Conclusions

Our results show that SERT deficiency strongly affected the emission of 22-kHz USV during differential fear conditioning. During acquisition, extinction, and recovery, SERT deficiency consistently led to a reduction in 22-kHz USV emission. While SERT deficiency did not affect immobility during acquisition, genotype differences started to emerge during extinction, and during recovery rats lacking SERT showed higher levels of immobility than wildtype littermate controls. Recovery was reflected in increased levels of immobility but not 22-kHz USV emission. Prominent sex differences were evident. Among several measures for trait-like inter-individual differences, anxiety-related behavior had the best predictive quality.

5. Materials and Methods

5.1. Animals and Housing

The effects of SERT deficiency on extinction and recovery in a differential fear conditioning paradigm were tested in male and female constitutive homozygous SERT$^{-/-}$ and heterozygous SERT$^{+/-}$ mutant rats, as compared to their wildtype SERT$^{+/+}$ littermate controls. SERT$^{-/-}$ rats completely lacking 5-HTT (SLC6A41Hubr) were generated by N-ethyl-N-nitrosurea (ENU) [101] and outcrossed with commercially available Wistar rats (Harlan, Ter Horst, The Netherlands) for at least 10 generations [21]. In total, N = 87 rats were included (N = 43 female rats (14 +/+, 15 +/−, 14 −/−), N = 44 male rats (15 +/+,

15 +/−, 14 −/−)). Rats were identified by paw tattoo and genotyping was performed as previously described [37].

To obtain SERT$^{-/-}$ and SERT$^{+/-}$ offspring together with SERT$^{+/+}$ littermate controls, a heterozygous breeding strategy was applied as before [37]. Briefly, female and male SERT$^{+/-}$ rats were paired for breeding. To avoid genetic drifts, male and female SERT$^{+/-}$ breeders were obtained by outcrossing SERT$^{+/-}$ males with Wistar females (Harlan, Ter Horst, The Netherlands). In order to avoid litter effects, only litters with all three genotypes were included in the experiments. After weaning on postnatal day 21, rats were socially housed in mixed-genotype groups of N = 4–5 with same-sex littermate partners in standard Macrolon Type IV cages with high stainless-steel covers (58 × 33 × 20 cm) and bedding in an animal room with a 12:12 h light-dark cycle (lights on from 7 a.m. to 7 p.m.). Standard rodent chow (Altromin, Lage, Germany) and water (0.0004% HCl solution) were available ad libitum.

5.2. General Procedure

Rats were tested with 2–4 months of age. After a standardized handling procedure on three consecutive days, the following behavioral assays were performed in the following order: activity box, elevated plus maze, novel object recognition, differential fear conditioning, and hot plate. The interval between behavioral assays was at least 2–3 days. Testing was conducted during the light cycle between 7 a.m. and 7 p.m. Equipment was thoroughly cleaned with a 0.1% acetic acid solution followed by thorough drying before each rat was tested. Rats were weighed after testing.

5.3. Activity Box

Novelty-seeking and habituation learning were assessed in an activity box, a small open field, as described previously [102]. The activity box (40 × 40 × 40 cm) was made of acrylic plastic and was equipped with an automated activity monitoring system (Tru Scan, Photobeam Sensor-E63-22; Coulbourn Instruments, Allentown, PA, USA). Activity box behavior was automatically monitored by means of two grids of infrared sensor beams mounted horizontally 2.5 cm and 14.5 cm above the floor for assessing distance travelled (in cm) and rearing behavior (number), respectively. The measure of rearing included all types of rearing, that is, irrespective of whether they were displayed on or off the walls. Testing began by placing the rat into a corner of the activity box, facing a wall. Activity box behavior was tested under red light (28 lx) conditions for 10 min on two consecutive days. Two and three rats were excluded from data analysis for the first and second day, respectively, due to data loss.

5.4. Elevated Plus Maze

Anxiety-related behavior was evaluated in an elevated plus maze, as previously described [102]. The apparatus was made of gray plastic and consisted of two opposed open arms and two opposed closed arms (arm sizes: 50 × 10 cm) extending from an open central square (10 × 10 cm). The maze was elevated 50 cm above the floor. Testing began by placing the rat into the center of the maze facing one of the open arms. Anxiety-related behavior was measured under conditions of white light (30 lx in the center) and videotaped using a digital camera (TVVR3304; ABUS, Affing, Germany). As parameter indicating anxiety-like behavior, time spent on open arms was analyzed using automated tracking software (Ethovison XT 14; Noldus, Wagenigen, The Netherlands). An open arm entry was defined as entry of the rat with all four paws including tail base. Overall locomotor activity was measured by means of distance travelled on the apparatus. Each rat was tested for 5 min on two consecutive days.

5.5. Novel Object Recognition

For assessing cognitive functioning, the novel object recognition test was conducted in a large open field made of gray plastic (60 × 60 × 60 cm), as previously described [103].

First, rats were habituated to the open field (no objects present) by placing them into the box for 10 min. Next, 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 (TVVR3304; ABUS, Affing, Germany) was mounted 1.5 m above the floor of the open field and connected to a personal computer for recording and data storage. Behavior was scored from video recordings by an experienced observer blind to the rat's genotype using The Observer XT (Noldus, Wagenigen, The Netherlands). 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 white light (40 lx) conditions.

5.6. Differential Fear Conditioning

5.6.1. Setup and Paradigm

Differential fear conditioning took place in a shock chamber (33.5 × 35 × 38 cm) made of gray and transparent plastic walls. The roof and one wall were made of transparent plastic to allow video observation during the test. A loudspeaker was mounted in one wall ~30 cm above the floor for presenting tones. The floor of the shock chamber was made of stainless-steel rods (diameter: 5 mm) spaced 1 cm apart. The chamber was placed in a sound attenuating isolation cubicle (51 × 71 × 51 cm; Coulbourn Instruments, Allentown, PA, USA) equipped with two white-light LED spots (~40 lx; Conrad Electronic, Hirschau, Germany) and a b/w CCD camera (Conrad Electronic, Hirschau, Germany) connected to a computer for videotaping. An UltraSoundGate Condenser CM 16 Microphone (Avisoft Bioacoustics, Berlin, Germany) was attached to the roof of the shock chamber ~30 cm above the floor. The microphone was connected via an UltraSoundGate 416 USB audio device to a 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 (Avisoft Bioacoustics, Berlin, Germany). The microphone is sensitive to frequencies of 15–180 kHz with a flat frequency response (±6 dB) between 25 and 140 kHz.

The differential fear conditioning paradigm consisted of three test phases: acquisition, extinction, and recovery. On the first day, day 1, acquisition was performed. The next day, day 2, extinction was tested. One week later, day 9, recovery was measured. Two distinct contexts were used. Context A was defined by lavender scent (0.2% solution; Primavera Life GmbH, Oy-Mittelberg, Germany) placed underneath the stainless-steel rods and visual cues made of 3.8 cm broad vertical white stripes (Tesa SE, Norderstedt, Germany) with 3.8 cm distance in between. Context B was defined by lemongrass scent (0.2 % solution; Primavera Life GmbH, Oy-Mittelberg, Germany) and visual cues made of 3.8 cm broad horizontal white stripes (Tesa SE, Norderstedt, Germany) with 3.8 cm distance in between. Rats were habituated to the contexts for 300 s.

Acquisition was performed in context A. During acquisition, rats were trained to associate an acoustic stimulus (conditioned stimulus plus, CS+) with electric shock (unconditioned stimulus, UCS), whereas a second stimulus was never paired with electric shock (conditioned stimulus minus, CS-). As CS, 2 kHz and 9 kHz sinewave tones (generated with: Avisoft SASLab Pro Synthesizer) were presented at 72 dB for 30 s. As USC, a 0.5 mA

scrambled shock (52 Hz, 120 V peak-to-peak amplitude; stand-alone shocker; Med Associates, St. Albans, USA) was used. The CS+ presentation was terminated by the electric shock of 500 ms duration. The CS- presentation was not terminated by an electric shock. Six presentations of CS+ and CS- each were applied in a pseudo-randomized, counter-balanced manner. Stimulus delivery and timing were controlled by the Presentation program (Neurobehavioral Systems, Albany, VT, USA). For acquisition, the rats were handled with gloves and carried to the testing apparatus on the arm of the experimenter.

Extinction was tested in context B the next day after acquisition. CS presentation (CS+/CS-) was pseudo-randomized and altered in comparison to acquisition. For extinction, the rats were handled without gloves and carried to the testing apparatus in a Macrolon Type II cage (27 × 22 × 14 cm).

Recovery was measured in context B on the seventh day after extinction. CS presentation (CS+/CS-) was pseudo-randomized and altered in comparison to acquisition and extinction. For recovery, the rats were handled identical to extinction, without gloves and carried to the testing apparatus in a Macrolon Type II cage (27 × 22 × 14 cm).

5.6.2. Analysis of Immobility

Immobility was scored in 30 s time bins from video recordings by an experienced observer blind to the rat's genotype using The Observer XT (Noldus, Wagenigen, The Netherlands), as previously described [37]. Immobility was defined as the suppression of all somatic motility except of motions associated with respiratory activity.

5.6.3. Analysis of Ultrasonic Vocalizations

The emission of 22-kHz USV was analyzed by an experienced observer blind to the rat's genotype using Avisoft SASLab Pro (Version 5.2.09; Avisoft Bioacoustics, Berlin, Germany), as previously described [37]. For acoustical analysis, 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). A lower-cut-off-frequency of 18 kHz was used to reduce background noise outside the relevant frequency band to 0 dB. Detection of 22-kHz USV was provided by an automatic threshold-based algorithm (threshold: −40 dB) and a hold time mechanism (hold time: 20 ms). Accuracy of 22-kHz USV detection was verified and a 100% concordance between automatic and observational detection was obtained. Total calling time was measured for entire test phases and separately for presentations of CS+ and CS-. As 22-kHz USV are emitted either as single pulses or in short bouts, calls were divided into those starting a bout versus those within a bout. Number of calls starting a bout and calls per bout were assessed.

5.7. Hot Plate

After differential fear conditioning, a hot plate test was performed under white light (~300 lx) to assess the effects of SERT deficiency on pain reactivity to thermal stimulation (precision hot plate, Prezitherm PZ35; Harry Geistigkeit GmbH, Düsseldorf, Germany). On the first day, the rat was placed onto the unheated apparatus for 120 s to habituate it to the test environment. On the next day, the rat was placed into the center of the hot plate kept at a constant temperature of 52 °C. The time to lick one of the four paws was measured by observation. To prevent tissue damage, a cut-off latency of 30 s was applied.

5.8. Statistical Analysis

All statistical tests were carried out using IBM SPSS Statistics (Version 25.0.0.1) software. To compare the body weight throughout different stages of the experiment, a repeated measures three-way ANOVA with the between-subject factors genotype (G) and sex (S) and the within-subject factor testing procedure was calculated, followed by repeated measures two-way ANOVAs separately for both sexes.

To assess differences in the prevalence of rats emitting 22-kHz USV during differential fear conditioning between experimental conditions, chi^2-tests were calculated. Overall immobility and 22-kHz USV emission were compared using two-way ANOVAs with the between-subject factors G and S. To determine differences in the reaction to CS+ and CS- presentations for the phases of extinction (EXT) and recovery (REC), repeated measures three-way ANOVAs with the between-subject factors G and S and the within-subject factor CS presentation (CS) were performed. The time course of immobility towards CS+ and CS- presentations was compared using a repeated measures four-way ANOVA with between-subject factors G and S and the within-subject factors of trial (TRIAL) in addition to CS. Single CS presentations at the beginning and the end of testing were compared using paired *t*-tests. As for the analysis of immobility throughout different days, the last CS presentations during EXT and the first during REC, respectively, were compared using a repeated measures four-way ANOVA with between-subject factors G and S and the within-subject factors of day (DAY) and CS.

Novelty-seeking in the activity box, anxiety-related behavior in the elevated plus maze, and pain sensitivity in the hot plate test were compared using two-way ANOVAs with the between-subject factors G and S. Habituation learning in the activity box was compared using a repeated measures three-way ANOVA with between-subject factors G and S and the within-subject factor DAY. Cognitive functioning in the novel object recognition test was analyzed with a repeated measures three-way ANOVA with between-subject factors G and S and the within-subject factor of object (OBJ), i.e., percentage of exploration time for familiar versus novel object. Paired *t*-tests were calculated to compare the percentage of exploration time for familiar versus novel object in the different experimental conditions. To correlate immobility displayed during differential fear conditioning, including acquisition, extinction, and recovery, with novelty-seeking, anxiety-related behavior, habituation learning, cognitive functioning, and pain sensitivity, Pearson correlation coefficients were calculated.

Since sphericity was not met for several repeated-measures ANOVAs, Greenhouse-Geißer corrected values are reported. ANOVAs were followed by post-hoc LSD tests when appropriate, i.e., following significant ANOVA results. Two-tailed significance threshold was set at 5%. All values are reported as mean and ±standard error of mean (SEM).

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ijms22137088/s1, Figure S1: CS+/CS- Presentation: immobility, last extinction vs. first recovery, grouped by genotype; Figure S2: CS+/CS- Presentation: 22-kHz USV, all rats; Figure S3: CS+/CS- Presentation: 22-kHz USV, grouped by genotype; Figure S4: novel object recognition. Figure S5: correlation, immobility, and 22-kHz USV.

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Supplementary figures

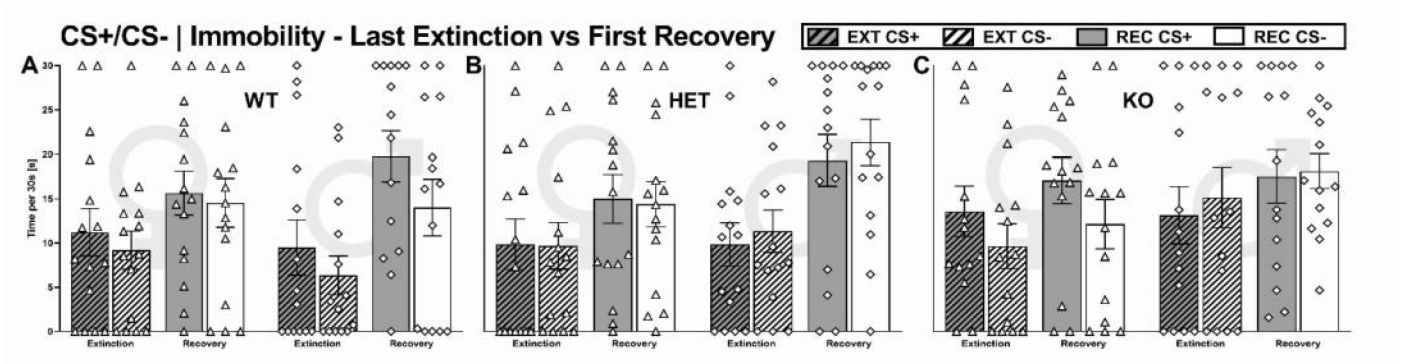

Figure S1. CS+/CS- Presentation | Immobility | Last Extinction vs First Recovery | Grouped by Genotype. Effects of SERT deficiency on immobility for the comparison of last trial extinction vs first trial recovery. Depicted are total amounts of immobility for last CS+ presentation (grey striped bar) and CS- presentation (white striped bar) during extinction (A, C), as well as CS+ presentation (grey bar) and CS- presentation (white bar) during recovery for $SERT^{+/+}$ (A), $SERT^{+/-}$ (B) and $SERT^{-/-}$ (C) rats. Two bars on the left comprise CS+ and CS- presentations for females; males are shown on the two right bars of every genotype. N = 29 $SERT^{+/+}$ (15 female, 14 male), 30 $SERT^{+/-}$ (15 female, 15 male), 28 $SERT^{-/-}$ (14 female, 14 male) rats. Data are presented as mean ± SEM.

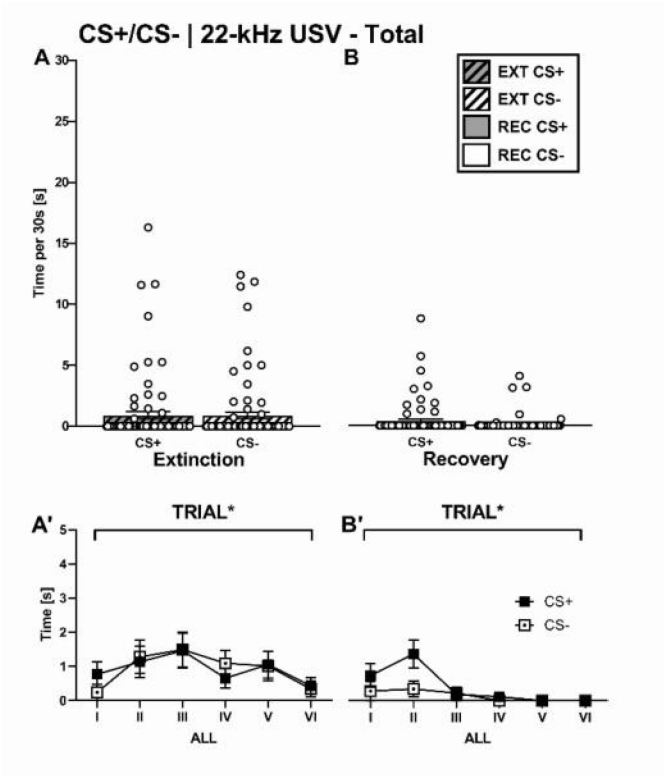

Figure S2. CS+/CS- Presentation | 22-kHz USV | All Rats. Effects of SERT deficiency on 22-kHz USV for CS+ and CS- presentations during extinction (A-A') and recovery (B-B'). Depicted are total amounts of 22-kHz USV for CS+ presentations (grey striped bar) and CS- presentations (white striped bar) during extinction (A), as well as CS+ presentations (grey bar) and CS- presentations (white bar) during recovery (B). Furthermore, single trial immobility levels for extinction (A') and recovery (B') are shown by means of CS+ presentations (black squares) and CS- presentations (white squares with dot). N = 87 rats. Data are presented as mean ± SEM. TRIAL* $p < 0.05$ effect of time

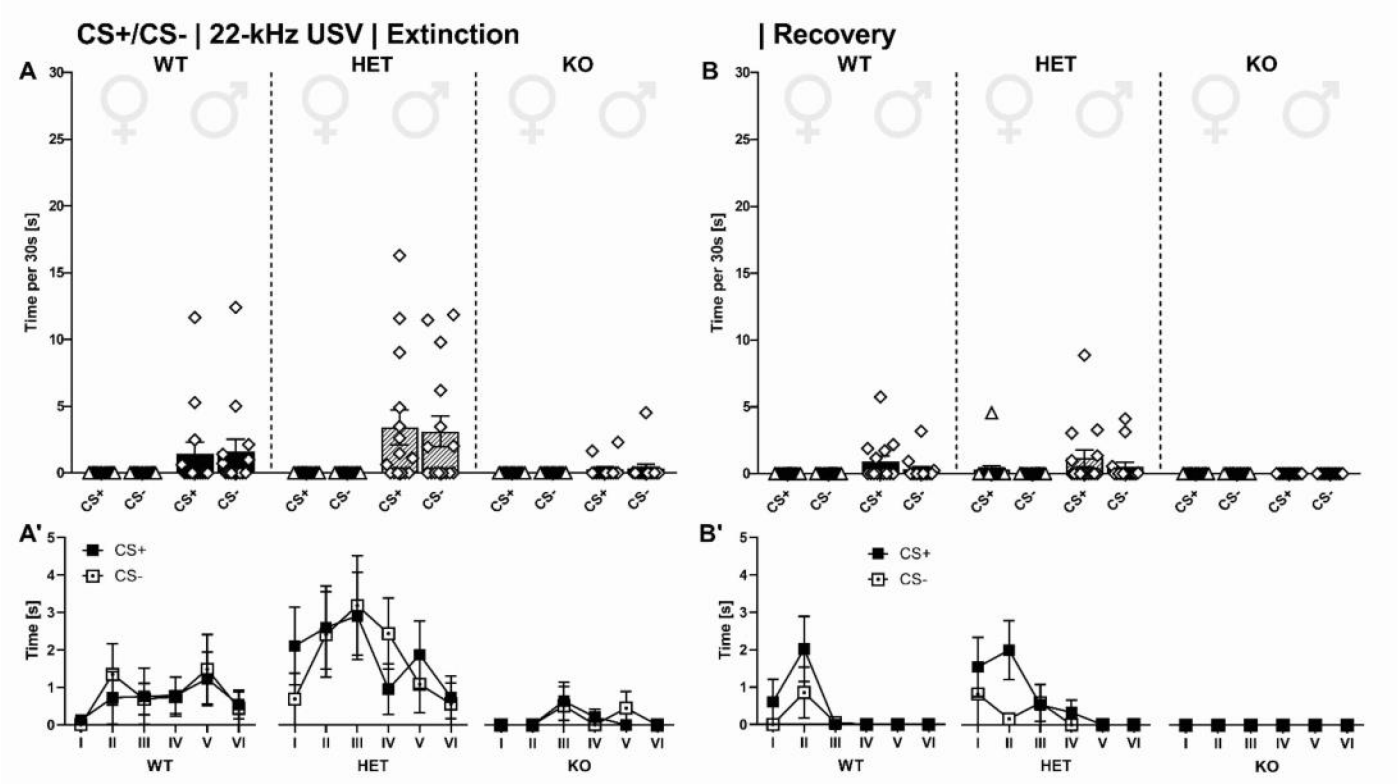

Figure S3. CS+/CS- Presentation | 22-kHz USV | Grouped by Genotype. Effects of SERT deficiency on 22-kHz for CS+ and CS- presentations during extinction (A-A') and recovery (B-B'). Depicted are total amounts of 22-kHz USV for both CS+ and CS- presentations for $SERT^{+/+}$ (black bar), $SERT^{+/-}$ (striped bar) and $SERT^{-/-}$ (white bar) rats. Two bars on the left comprise CS+ and CS- presentations for females; males are shown on the two right bars of every genotype. Furthermore, single trial immobility levels for extinction (A') and recovery (B') are shown by means of CS+ presentations (black squares) and CS- presentations (white squares with dot). N = 29 $SERT^{+/+}$ (15 female, 14 male), 30 $SERT^{+/-}$ (15 female, 15 male), 28 $SERT^{-/-}$ (14 female, 14 male) rats. Data are presented as mean ± SEM.

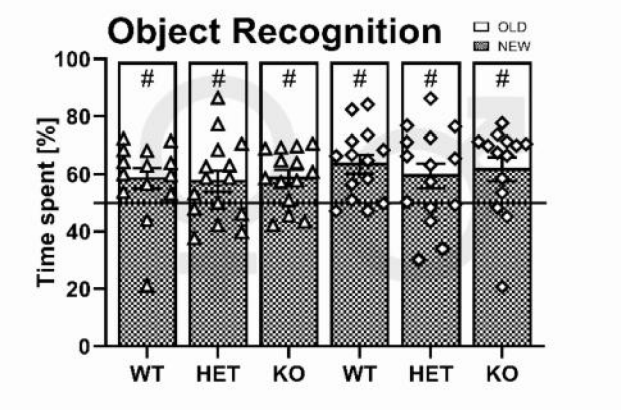

Figure S4. Novel Object Recognition. Effects of SERT deficiency on cognitive functioning. Depicted is percentage of time spent exploring a familiar object (old, white bar) vs a novel object (new, checkered bar) for $SERT^{+/+}$, $SERT^{+/-}$ and $SERT^{-/-}$ female rats (three left panels) and their male conspecifics (three right panels). N = 44 female rats (15 +/+, 15 +/-, 14 -/-), N=43 male rats (14 +/+, 15 +/-, 14 +/-). Data are presented as mean ± SEM. # $p < 0.05$ significant within-subject comparison of familiar vs novel object.

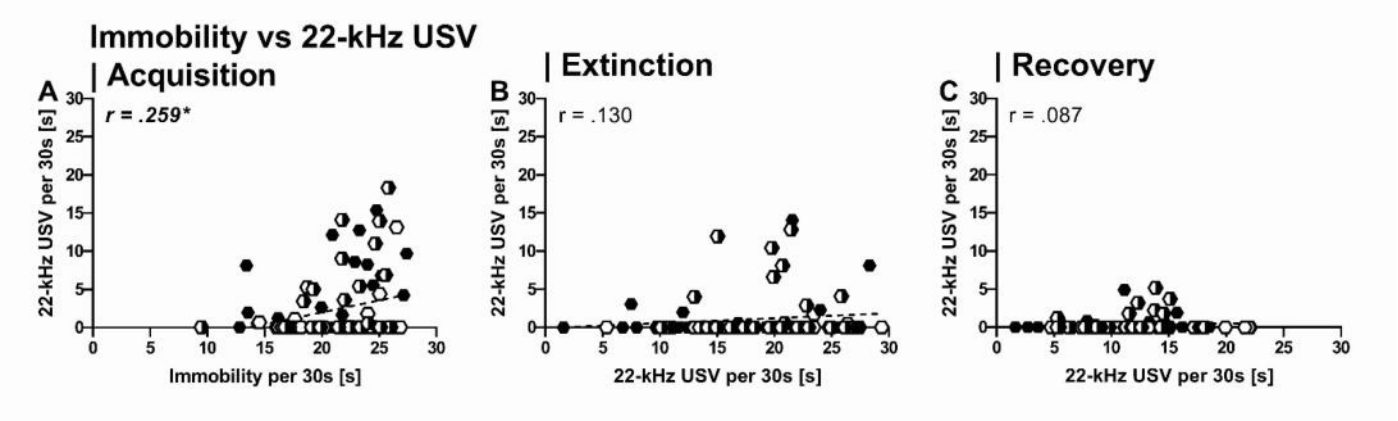

Figure S5. Correlation – Immobility and 22-kHz USV. Duration of immobility in relation to 22-kHz USV during acquisition (A), extinction (B), and recovery (C). Depicted are SERT$^{+/+}$ (black diamond), SERT$^{+/-}$ (black-white diamond), and SERT$^{-/-}$ (white diamond) rats. N = 29 SERT$^{+/+}$ (15 female, 14 male), 30 SERT$^{+/-}$ (15 female, 15 male), 28 SERT$^{-/-}$ (14 female, 14 male) rats. Data are presented as individual values and correlation coefficients. Statistical significance ($p < .05$) of correlation coefficient is indicated in ***bold and italic***.

6 ANHANG

6.1 Abkürzungsverzeichnis

5-HT	**Serotonin**
5-HTT	Serotonintransporter
5-HTTLPR	*serotonin-transporter-linked promoter region*; Serotonin-Transporter-Promoter-Polymorphismus
8-OHDPAT	8-hydroxy-2-(di-n-propilamino)tetralin
BLA	basolaterale Amygdala
BNST	*bed nucleus of the stria termalis*; Nucleus striae terminalis
CeA	*central amygdala*; zentrale Amygdala
CR	*conditioned reaction*; konditionierte Reaktion
CRH	Corticotropin-Releasing-Hormon
CS	*conditioned stimulus*; konditionierter Stimulus
DS	*danger stimulus*, Gefahrensignal
EPM	*elevated plus maze*; erhöhtes Plus-Labyrinth
FM	Frequenzmoduliert
HC	*high calling*
HOA	*high open arm*
HPA-Achse	Hypothalamus-Hypophysen-Nebennierenrinden-Achse
LC	*low calling*
LOA	*low open arm*
mCPP	meta-Chlorophenylpiperazine
NPS	Neuropeptid S
PAG	Periaquäduktales Grau
PFC	Präfrontaler Kortex
PTBS	Posttraumatische Belastungsstörung
PTZ	Pentylenetetrazol
SERT	Serotonintransporter
SERT-HET	heterozygoter Serotonintransporter Knockout
SERT-KO	homozygoter Serotonintransporter Knockout
SERT-WT	Serotonintransporter Wildtyp
SS	*saftey stimulus*, Sicherheitssignal
SSRI	Selektive Serotonin-Wiederaufnahme-Inhibitoren
TMT	2,N,N-trimethyltryptamine
US	*unconditioned stimulus*; unkonditionierter Stimulus
USV	Ultraschallvokalisationen

6.2 Abbildungs- und Tabellenverzeichnis

7 LITERATURVERZEICHNIS

[A]

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9 CURRICULUM VITÆ

[Die Seiten 191-193 (Curriculum vitæ) enthalten persönliche Daten. Sie sind deshalb nicht Bestandteil der Online-Veröffentlichung.]

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ERKLÄRUNG

Ich versichere, dass ich meine Dissertation

ANGST UND FURCHT IN DER RATTE

— über den Einfluss situationsspezifischer und biologischer Faktoren auf das Spektrum der Ultraschallvokalisationen —

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