Playback of ultrasonic vocalizations in rats: habituation, response calls, and drug effects

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“I am among those who think that science has great beauty.”

-Marie Curie-

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Summary

Rats are highly social animals. They have developed a variety of social behaviors including communication via so-called ultrasonic vocalizations (USV). Among these USV, two types can be distinguished in juvenile and adult rats. Appetitive 50-kHz USV are thought to represent a positive affective state, whereas aversive 22-kHz USV are supposed to depict a negative affective state. Playback of positive 50-kHz USV induces an approach behavior in rats as seen by their approach behavior to the sound source. Previous studies have shown that this behavior is only detectable during the first presentation, whereas a quick habituation towards 50-kHz USV results in the rats not approaching the 50-kHz USV playback a second time, even after several days. This habituation phenomenon seems to rely on learning and memory mechanisms. However, its underlying mechanisms have been studied scarcely so far. This dissertation revealed three factors influencing the habituation phenomenon. First, habituation was dependent on stocks. It was only present in Wistar but not Sprague-Dawley rats . Second, habituation could be prevented with treatment of the dopaminergic agonist d-amphetamine before the second 50-kHz USV playback. Third, habituation was state-dependent. It was shown that when the pharmacologically induced internal state changed between the two playbacks, no habituation occurred.

Furthermore, the reciprocal nature of USV was investigated in this dissertation. Calls in response towards 50-kHz USV playback had been reported before, but this dissertation is the first to characterize response calls. We showed that response calls towards 50-kHz playback are around frequencies of 30 kHz, have a rather short duration of 0.3 s and hardly any frequency modulation. These parameters resemble aversive 22-kHz calls, which are unlikely to be found in an appetitive paradigm as the 50-kHz USV playback. Feasible functions of these response calls might be a frustrated state due to expectation violation after playback, appeasement calls to pacify the potential play partner indicated by the playback, or they might serve as social contact calls to establish proximity.

Taken together, the findings of this dissertation shed light on the reciprocal nature of USV communication indicated by response calls towards 50-kHz USV playback and present possible mechanisms how to overcome the habituation phenomenon. This provides tools to further investigate neurodevelopmental disorders where communication and social behavior is impaired, such as autism spectrum disorder or the Angelman Syndrome, as well as affective disorders.

Zusammenfassung

Ratten sind äußerst soziale Tiere. Dadurch haben sie eine Vielzahl von Sozialverhalten entwickelt, unter anderem die Kommunikation mittels so genannter Ultraschallvokalisationen (USV). Bei diesen USV können in juvenilen und erwachsenen Tieren zwei Klassen unterschieden werden. Appetitive 50-kHz USV sind wahrscheinlich der Ausdruck eines positiven affektiven Zustandes, wohingegen aversive 22-kHz USV womöglich einen negativen affektiven Zustand darstellen. Playback von positiven 50-kHz USV induzieren ein Annäherungsverhalten bei Ratten, was durch die Annäherung an die Schallquelle zu verzeichnen ist. Frühere Studien haben gezeigt, dass dieses Annäherungsverhalten nur während eines ersten Playbacks zu sehen ist, wohingegen eine schnelle Gewöhnung an 50-kHz USV stattfindet, sodass die Ratten sich dem 50-kHz USV Playback kein zweites Mal nähern, auch nicht Tage später. Dieses Gewöhnungs-Phänomen scheint auf Lern- und Gedächtnismechanismen zu beruhen. Jedoch wurden die zugrundeliegenden Mechanismen bisher kaum untersucht. Diese Dissertation zeigte drei Faktoren, die das Gewöhnungs-Phänomen beeinflussen. Erstens, ist die Gewöhnung abhängig vom Rattenstamm. Sie war nur in Wistar, jedoch nicht in Sprague-Dawley Ratten zu verzeichnen. Zweitens, konnte die Gewöhnung mittels Behandlung des dopaminergen Agonisten d-Amphetamin vor dem zweiten 50-kHz USV Playback verhindert werden. Drittens, ist die Gewöhnung abhängig vom Zustand der Tiere. Es konnte gezeigt werden, dass die pharmakologische Induktion eines inneren Zustandes die Gewöhnung verhinderte, soweit sich der Zustand zwischen den beiden Playbacks unterschied.

Zusätzlich wurde die Wechselwirkung von USV in dieser Dissertation untersucht. Rufe als Antwort auf das 50-kHz USV Playback wurden zwar schon früher berichtet, jedoch ist diese Dissertation die erste, die die Charakteristiken dieser Antwortrufe genauer beschreibt. Es konnte gezeigt werden, dass Antwortrufe gegenüber 50-kHz Playback Frequenzen von etwa 30 kHz besitzen, eine eher kurze Dauer von 0.3 s haben und kaum Frequenzmodulationen aufweisen. Diese Parameter ähneln aversiven 22-kHz Rufen, welche unwahrscheinlich in appetitiven Paradigmen wie dem 50-kHz USV Playback zu finden sein sollten. Mögliche Funktionen dieser Antwortrufe könnten ein frustrierter Zustand sein, der durch die Erwartungsverletzung nach dem Playback zustande kommt. Weitergehend könnten sie der Beschwichtigung eines potentiellen Spielpartners, angezeigt durch das Playback, dienen. Darüber hinaus könnten Antwortrufe auch als soziale Kontaktrufe dienen, um Nähe herzustellen.

Zusammengefasst zeigen die Ergebnisse dieser Dissertation die Wechselwirkung von USV Kommunikation, dargestellt durch Antwortrufe gegenüber 50-kHz USV Playback. Außerdem werden Möglichkeiten aufgezeigt, welche Mechanismen das Phänomen der Gewöhnung überwinden können. All dies bietet Perspektiven, um Entwicklungsstörungen mit eingeschränkter Kommunikation und Sozialverhalten, wie Autismus-Spektrum-Störungen oder das Angelman-Syndrom, so wie affektive Störungen zu untersuchen.

Introduction

Communication: a brief overview

Communication, noun, /kəˌmjuːnɪˈkeɪʃ(ə)n/

The activity or process of expressing ideas and feelings or of giving people information (Hornby et al., 2000: Oxford Advanced Learner's Dictionary).

From the Latin word communis, common (Schramm & Roberts, 1971).

In its original form, communication is a transmission of information from one to one another, or the understanding of a difference of utterance and information (Luhmann, 1992). Communication has long been not only necessary for survival, but humankind has also embraced it as art in written and oral form, as well as in performance and for entertainment (Hovland, 1948). Among the animal kingdom, various species have developed numerous types of communication, most of them being of vocal nature and serve as a key function for mating, survival, and socialization. Especially among species, living in social constructs (Hauser, 1996; Bradbury & Vehrencamp, 2011).

Well-known scientists like Karl von Frisch, Konrad Lorenz, and Niko Tinbergen have set cornerstones for the research field of animal communication and provided groundbreaking work to understand animals' behavior, the innateness of behavior, and why animals act like they do (Lorenz, 1935; von Frisch, 1974; Tinbergen, 1952). Communication does not necessarily involve individuals of one specific species, but the exchange of information between different species is possible, like Lorenz demonstrated in his studies about imprinting in geese. In one of his experiments, Lorenz himself was the first object the newborn geese saw and thus they were imprinted on him and followed him wherever he went (Lorenz, 1935). Karl von Frisch did marvelous research on the communication between bees and characterized the tail wagging dance that makes it possible to communicate the exact location of a feeding place in respect to the sun's position from one bee to the other worker bees in the hive (von Frisch, 1974). Niko Tinbergen wrote about innate behavior and instincts, which serves as the foundation for the modern cross section of behavior and genes. Without their work, nowadays research about behavior, genes, the brain, neuropsychiatric disorders, and overall, about communication would not be possible.

Studies about communication date back to early 1940s, where Hovland (1948) already defined four parts of communication: the communicator, the stimuli that are transmitted, the individuals that respond, and the responses themselves. These levels can also be transferred to the field of animal studies in this dissertation, which would be the sender, the transmitted stimuli, the receiver, and the responses.

Further on scientists discovered ultrasonic vocalizations (USV; >20 kHz, see next section) as a way of communication in mammalian species like bats, dolphins, and rodents (Bats: Pierce & Griffin, 1938; dolphins: Kellogg et al., 1953; Sales et al., 1974; Goldstein & Brockmole, 2016) or even in insects (Pierce, 1948) or amphibians like frogs (Feng et al., 2006).

Gould and Morgan (1941) were the first ones to identify the rat's hearing ability of up to 40 kilocycles (equivalent to 40 kHz nowadays). Because their apparatus only allowed measurements of up to 40 kHz. Later on, the first scientific characterization and definition of USV in rodents and other mammal species was done by Anderson in 1954 (Anderson, 1954). In these first experiments about USV in other mammals than bats, Anderson used a "sonic amplifier" and identified the emission of rat calls as high as 80 kHz.

Nowadays numerous research facilities around the world are using the techniques of quantifying USV as a readout during various experiments to investigate their function and use (Panksepp & Burgdorf, 2000; Knutson et al., 2002; Scattoni et al., 2009; Wöhr & Schwarting, 2013). Early on, USV emission and affect were linked together by showing that USV emitted by pups can induce retrieval behavior in the dam (Sewell, 1970; Zippelius & Schleidt, 1970). Since then, not only rat models but also mouse models have been used for researching socio-affective communication via USV. Basic research has profited profoundly from these findings by understanding neural mechanism of the production as well as receiving USV (Wöhr & Schwarting, 2013). Most importantly, several models for neuropsychiatric disorders, where socio-affective communication is impaired, have been developed to understand the underlying genetics and neural mechanisms and to find cures against disorders such as affective disorders, schizophrenia, neurodevelopmental disorders, or addiction (Jones et al., 2011; Wöhr & Scattoni, 2013; Servadio et al, 2015; Spanagel, 2017; Kisko et al., 2018; Berg et al., 2020; Wöhr et al., 2021).

Ultrasonic vocalizations

Ultrasonic vocalizations are defined as frequencies above 20 kHz, which is outside the human hearing range. USV are emitted by various species such as whales, dolphins (Madsen and Surlykke, 2013; McGowen et al., 2014), or bats (Jones and Teeling, 2006; Tzu et al., 2015) but as well in rodents like mice (Wöhr & Scattoni, 2013; Portfors and Perkel 2014) and rats (Brudzynski, 2013; Wöhr & Schwarting, 2013).

In rats, USV have been described as situation-dependent socio-affective signals (Wöhr, 2018), which can be divided into three main categories with distinct frequencies: isolation-induced 40-kHz pup vocalizations, 50-kHz appetitive USV, and 22-kHz aversive USV. These three categories have different specific features to them such as mean peak frequency, call duration, or frequency modulation, as well as different functions.

Isolation-induced 40-kHz pup vocalizations

Forty-kilohertz ultrasonic vocalizations are emitted by pups when they are separated from their mother and the littermates. These calls are also known as isolation-induced USVs because they mainly occur during a separation of pups from the dam from postnatal day (PND) one to approximately PND 23 (Nitschke et al., 1975; Hofer et al., 1998; Cox et al., 2012). The frequency range of these USV was reported to be between 20 to 90 kHz (Nitschke et al., 1975; Cox et al., 2012), whereas most calls are between 30 and 60 kHz (Shair, 2018) and their relatively short duration is around 80-150 ms (Brudzynski et al., 1999; Schwarting & Wöhr, 2012). A comparison between pups of three different rat stocks, namely Wistar, Sprague-Dawley, and Long Evans rats, showed that call numbers as well as several call features differed between the stocks (Schwarting & Wöhr, 2018). This was tested by keeping the pups individually isolated for 10 min on PND 11. Sprague-Dawley pups emitted fewer calls with lower peak frequencies and less frequency modulation compared to Long Evans and Wistar rats. Additionally, three different call types that differed in mean peak frequencies and frequency modulation, and duration were identified. The proportions of these types differed between the stocks with calls or low peak frequencies (~40 kHz) and small modulation (~4 kHz) being least prominent in Long Evans rats (Schwarting & Wöhr, 2018).

Putting the rodents outside their nest, the emission of the 40-kHz USV induces retrieval behavior in the dam, whose hearing ability is most sensitive in the range around 40 kHz. The retrieval by the mother is life saving and without it the pups would die from hypothermia and starvation (Allin & Banks, 1972; Wöhr et al., 2010). It is argued whether the 40-kHz isolation-induced USV represent a negative effective state, distress, or anxious behavior, or if they are simply a byproduct of the cooling effect (Schwarting & Wöhr, 2012). Also, other rodent species, like mice and voles, emit these calls when they are put outside their nest. Early studies called them “whistles of abandonment” (Pfeifen des Verlassenseins) as K. Lorenz had termed similar vocalizations previously in geese pups (Lorenz, 1935; Zippelius and Schleidt, 1956).

50-kHz appetitive vocalizations

Fifty-kilohertz ultrasonic vocalizations are thought to represent a positive affective state (Panksepp & Burgdorf, 2000; Wöhr & Schwarting, 2013). Mean peak frequencies of these calls range between 35 to 90 kHz with relatively short durations around 10 to 30 ms and a bandwidth of 5 to 7 kHz (Simola & Brudzynski, 2018a). They are mainly emitted in positive situations like feeding (Knutson et al., 2002; Opiol et al., 2015; Simola & Brudzynski, 2018b), mating (Sales, 1972; Burgdorf et al., 2008), or play behavior (Knutson et al., 1998; Webber et al, 2012). In earlier studies 50-kHz USV were characterized as “rat laughter” (Panksepp, 2005) because they occurred during heterospecific play, i.e. tickling (Panksepp & Burgdorf, 2000). During tickling, an experienced experimenter is mimicking movements and behavior, like “belly-tickling” and “grab and tickle", that otherwise a littermate would do during rough-and-tumble play (Panksepp & Burgdorf, 2000; Burgdorf et al., 2005; Schwarting et al., 2007).

For analyses and categorization of results during behavioral paradigms, mostly four sub-categories of 50-kHz USV can be defined into flat calls and frequency modulated calls, with step, trill, and mixed calls as defined by Pereira and colleagues (Pereira et al., 2014). Flat calls were characterized as calls with frequency modulations below 5 kHz, step-calls with a flat element that had to include a step of at least 5-kHz frequency difference to the next call component, trills with a peak frequency of at least 5 kHz or with more frequency changes into opposite directions, and mixed calls that could not be categorized as none of the previous categories and, for example a mix of a trill and a flat component (Pereira et al., 2014). This categorization is much more convenient for analyses of behavioral paradigms, since it depicts and comprises all the important call types necessary for describing and comparing behavioral effects (Kisko et al., 2018). However, 50-kHz USV can further be divided up to 14 sub-categories. Wright et al. (2010) were the first to define these sub-categories ranging from flat 50-kHz calls, over different step and ramp calls with less frequency modulation to complex, trill and composite calls with higher frequency modulations (Wright et al., 2010). Additionally, to defining the sub-categories, they tested the effect of the dopaminergic agonist amphetamine (AMPH) on USV emission. AMPH increased the number of emitted 50-kHz USV and also altered the call profile with trills increasing dose-dependently indicating that the dopaminergic system is involved in 50-kHz USV emission (Wright et al., 2010).

Furthermore, it was shown on a cellular and physiological level that the dopaminergic reward system is involved in receiving and emitting 50-kHz USV via for example fast-scan cyclic voltammetry (Brudzynski, 2013; Willuhn et al., 2014). Willuhn et al. showed an increase of phasic dopamine release in the nucleus accumbens (NAc) during 50-kHz USV playback, which induces a strong and reliable approach behavior in rats (Wöhr & Schwarting, 2007, 2009; Seffer et al., 2014; Willuhn et al., 2014; Berg et al., 2018, 2020). Via immunohistology and marking the immediate early gene c-fos, an increased activity of the NAc could also be detected, which is an important area of the limbic system and part of the reward system (Sadananda et al., 2008). Other cellular methods, like single cell recordings, proved a decreased activity of the amygdala during playback of 50-kHz USV (Parsana et al., 2012).

The important role of 50-kHz USV in social communication and maintaining social structures becomes even more obvious in juvenile play behavior, where a wide variety of 50-kHz could be detected (Knutson et al. 1998; Lukas & Wöhr 2015). Different types of 50-kHz calls were even associated with distinct behaviors during play and to ensure that the interaction remains playful without escalating to aggression (Kisko et al., 2016; Burke et al., 2017a, b, 2020). Burke et al.

(2017b) also found that 50-kHz calls used during play can be categorized into 3-4 sections based on the situation they are used in. For example, composite and multi-step calls were predominantly correlated with running and jumping, whereas calls with trills were associated with slower movements (Burke et al., 2017b). Also, they doubted the hypothesis that 50-kHz calls are only a byproduct of movement by the physical compression of thorax, which was stated in early USV studies (Thiessen & Kittrell, 1979; Blumberg, 1992) and rather suggested USV are coordinating "moment-to-moment social interactions" (Burke et al., 2017b).

Since 50-kHz USV are believed to represent a positive affective state, analysis of this call type can serve as behavioral readout also in animal models for neuropsychiatric diseases (Kisko et al., 2018; Braun et al., 2019; Berg et al., 2021a). In a *Ube3a* deletion rat model of the Angelman Syndrome (AS) an excessive laughter-like vocalization behavior of the rats was observed as an indicator of a happy demeanor as seen in individuals with AS, among other symptoms including impaired cognition or aberrant social interactions (Berg et al., 2021a). In another model for the cross-disorder risk gene *CACNA1C* it was shown that haploinsufficiency of this gene, with only one parental copy of the gene, leads to less emission of 50-kHz USV during rough-and-tumble play and also reduced social approach towards 50-kHz USV playback suggesting an important role in socio-affective communication as observed in affective disorders like bipolar disorder, schizophrenia, or autism spectrum disorder (Kisko et al., 2018).

22-kHz aversive vocalizations

Twenty-two kilohertz calls presumably represent a negative affective state. These USV are emitted during aversive situation with external danger, as presence of a predator or only exposure of predator's scents (Blanchard et al., 1991; Fendt et al., 2018), or during a frustrated or dysphoric state without external danger (Simmons et al., 2018). The mean peak frequencies are defined between 18 and 32 kHz with either short durations below 0.3 s or long durations up to 3 s (Brudzynski et al., 1993). Frequency modulations are usually very low, since most 22-kHz calls are constituted of one flat component (Blanchard et al., 1991; Brudzynski & Ociepa, 1992; Brudzynski, 2001; Brudzynski & Holland, 2005; Simola & Brudzynski, 2018a). With low frequencies, these USV are far-reaching and especially good to communicate, for example, an approaching predator within a borrow (Blanchard et al., 1991). These calls are not only used in nature to communicate among the rats. Also, in laboratory settings 22-kHz calls were shown during stressful paradigms, such as fear conditioning with administration of electric shocks (Cuomo et al., 1988; Rowan et al., 1990; Molewijk et al., 1995). Among 22-kHz USV, long (>300 ms) and short (<300 ms) calls are distinguished (Brudzynski et al., 1993). Long calls are emitted during aversive situation with external danger, like predator exposure, post-ejaculatory refractory time, or in situations the animals associate with previous aversive situations, e.g. during fear conditioning (Barfield & Thomas, 1986; Cuomo et al., 1988; Blanchard et al., 1991; Simmons et al., 2018). Short 22-kHz USV, however, are much more ambiguous and it is not postulated yet in which exact situations they occur (Brudzynski, 2021). Short 22-kHz calls were suggested to appear during stressful situations without external influence to represent a negative affective state, i.e. frustration (Taylor et al., 2019) or during drug withdrawal (Ma et al., 2010; Simmons et al., 2018). In addition, during investigative situations and stimulus-seeking behavior this type of call was also shown besides flat 50-kHz calls (Robakiewicz et al., 2019). During playback studies, the emission of 22-kHz USV was also reported in several studies including Study II presented in this dissertation (Wöhr & Schwarting, 2007, 2009; Willadsen et al., 2014; Willuhn et al., 2014; Engelhardt et al., 2017, 2018; Berg et al., 2018, 2021a; Kisko et al., 2020; Olszyński et al., 2020; 2021; Berz et al., 2022). The calls were mainly in frequency ranges of 22-kHz USV, but shorter in duration. This led to the conclusion that the found and characterized response calls towards 50-kHz USV playback serve as a kind of contact calls, resemble short 22-kHz calls and might represent an aversive state of frustration (Berz et al., 2022). In this context, Burke et al. (2017a) showed calls of similar frequencies, around 20-30 kHz, with a flat component being important in situations that were at risk to escalate into aggression during play fighting with higher frequencies usual for 22-kHz USV, with some frequency modulation, which they called trill component (Burke et al., 2017a). It has been postulated that the emission of 22-kHz USV is not innate but has to be learned and associated with aversive situations (Endres et al., 2007; Bang et al., 2008; Wöhr & Schwarting, 2010). For example, Endres et al. (2007) showed that rats were able to learn the association between 22-kHz USV and an aversive situation, and later Bang et al. (2008) postulated that freezing behavior during 22-kHz emission is "a consequence of associative learning" (Endres et al., 2007; Bang et al., 2008). However, the behavior that is typically paired with the emission of 22-kHz calls in juvenile or adult rats is freezing or behavioral inhibition or even avoidance behavior (Brudzynski & Chiu, 1995; Fendt et al, 2018). Therefore, it is often measured in aversive experimental situations like fear conditioning or playback of 22-kHz USV (Wöhr & Schwarting, 2008; Fendt et al., 2018; Willadsen et al., 2021). A correlation of the emission of 22-kHz calls and freezing behavior has been shown in many studies, also in knockout models, such as serotonin transporter 5-HTT knockout model (Willadsen et al., 2021). The underlying neuronal mechanisms of 22-kHz USV predominantly involve the cholinergic system, which is also strongly linked to anxiety and fear (Brudzynski, 2014; Brudzynski et al, 2018). In playback studies, it has been found that brain regions in the periaqueductal gray, the perirhinal cortex, and the amygdala are active during hearing 22-kHz USV playback (Sadananda et al., 2008). Moreover, electrophysiological studies showed an increased activity in the amygdala during the presentation of 22-kHz calls, supporting the link to anxiety and fear (Parsana et al., 2012). The emission of 22-kHz USV can also be induced by pharmacological applications of cholinergic agonists, such as carbachol, another indicator that the serotonergic system is involved in 22-kHz USV production

(Brudzynski, 1994). Studies with a knockout of the serotonin transporter 5-HTT, for example, showed that a full knockout of the transporter decreases 22-kHz USV emission during a fear conditioning paradigm compared to wildtype littermates. Especially in females, this was prominent, showing a sexual dimorphism, which underlines the involvement of the serotonergic system (Willadsen et al., 2021).

Contact and response calls

As mentioned above, communication defines a key function for mating, sociability, and survival and guarantees maintaining social bounds and constructs. To communicate within these bonds and constructs the sender has to emit specific signals to establish contact. Therefore, many species emit so called contact calls.

Contact calls exist in many species of birds and mammals. Whereas there is a great body of literature about birds' songs, less is known about calls. A call differs from a song by its genetic predetermination and that it does not have to be learned extensively (Marler, 2004; Kondo & Watanabe, 2009). Among contact calls there are two differentiations: the agonistic and the affiliative call. Agonistic calls are for interaction between conspecifics as well as strangers; whereas affiliative calls are oriented only towards conspecifics like mating partners or group members. Affiliative contact calls are supposed to maintain bonds between conspecifics and contain social information about the sender. This information can be about identity (mate or kin), group membership, distance, or function (Marler, 2004; Kondo & Watanabe, 2009). One of the most popular contact calls containing information about identity are probably the "signature whistles" of bottlenose dolphins. These calls represent the acoustic contour specific to one individual (Janik & Slater, 1998). A recent study showed that such a vocal signature exists in rats as well (Vielle et al., 2021). Essentially, a significant feature of acoustic calls is the ability to transmit information over longer distances and even over physical barriers (Bradbury & Vehrencamp, 1998). This makes it possible to transfer important information from a sender to recipient.

Experimental approaches of contact calls include playback studies for example in bottlenose dolphins and macaques (Caldwell & Caldwell, 1965, 1968; Janik & Slater, 1998). When playing back distress calls of female macaques to males, the response was stronger towards playback of calls of friends compared to non-friends (Lemasson et al., 2008; Fischer et al., 2013). Furthermore, young Barbary macaques responded stronger to playback of their mother's calls compared to calls of strangers (Fischer, 2004; Fischer et al., 2013). This shows that contact calls can help to differentiate the receiver's response depending on whether it is a known conspecific or a stranger. The phenomenon of habituation towards contact calls has also been demonstrated in Barbary macaques (Fischer, 1998). The results of that study showed that monkeys categorize calls similar to humans (Fischer, 2013). Also, Hammerschmidt et al. (2009) found that male contact calls, also called songs, could elicit approach behavior and quick habituation towards the calls in female mice.

Contact calls in rodents

In rodents, 50-kHz USV have been postulated to serve as situation-dependent socio-affective signals being important for behaviors like play or mating (Wöhr, 2018). Several studies have led to the conclusion that these calls function as social contact calls and/or play signals. Therefore, researchers, for example, conducted different breeding studies, where they selectively bred for rats with high emissions of 50-kHz USV during tickling, which is imitated rough-and-tumble play by a human experimenter (Panksepp & Burgdorf, 2000; Panksepp et al., 2001; Burgdorf et al., 2005, 2009). It became evident that rats from the high emission line indeed emitted more 50-kHz USV in response to tickling (Panksepp et al., 2001; Burgdorf et al., 2005). Another indicator that 50-kHz USV are play signals that have to be learned was shown in devocalizing studies with rats (Panksepp et al., 2002; Panksepp & Burgdorf, 2003; Himmler et al., 2014; Kisko et al., 2015a, b). Pairs where both play partners were devocalized exhibited less rough-and-tumble play than in intact pairs and pairs, where the rats had been housed together with devocalized rats also showed less play behavior (Kisko et al., 2015b). These findings suggest that 50-kHz USV are important to engage in playful behavior and maintaining it likely by signaling a positive affective state to maintain the social interaction.

In line with the idea that 50-kHz USV serve as social contact calls in rodents is that animals prefer to spent more time in proximity of conspecifics that emit a high number of 50-kHz USV (Panksepp et al., 2002). Also, experiments, where the environment contained odors of previously removed conspecifics led to emission of 50-kHz USV in the tested rat. The amount of emitted USV was positively correlated with the number of rats leaving their odor (Brudzynski & Pniak, 2002).

In addition to the findings that underline the idea of 50-kHz USV being social contact calls, which is also represented by social approach behavior during playback, response calls towards 50-kHz USV playback have been reported in several studies (Wöhr & Schwarting, 2007, 2009; Willadsen et al., 2014; Willuhn et al., 2014; Engelhardt et al., 2017, 2018; Berg et al., 2018, 2021a; Kisko et al., 2020; Olszyński et al., 2020; 2021; Berz et al., 2022). This reveals the reciprocal nature of acoustic communication and that a signal from a sender elicits a response in the receiver. Even though such response calls towards 50-kHz USV playback have been reported in several studies, still little is known about these calls. Response calls were seen in both sexes (Berg et al., 2018, 2021a) but the emission of calls in response to 50-kHz USV playback was found to be more prominent in males than females in one study (Kisko et al., 2020). Again, as in the mentioned contact calls, age is possibly another important factor, because juvenile rats seem to emit more response calls compared to adults (Wöhr & Schwarting, 2009). Additionally, earlier experiences

(Olszyński et al., 2021) and inter-individual differences (Engelhardt et al., 2018) might be important components for the emission of response calls. Nevertheless, a detailed study about response calls towards 50-kHz USV playback has not been conducted yet, which is why we performed Study II of this thesis, to characterize these calls and reveal possible functions of response calls. In this study, we came to the conclusion that response calls towards 50-kHz USV playback do not fit into the already known USV categories of 22- and 50-kHz calls. We hypothesized that response calls, besides the hypotheses of representing an ambivalent or even frustrated state, or functioning as appeasing calls, might serve as contact calls (Berz et al., 2022).

Social behavior

Social, adjective, \ ˈsō-shəl \:

Relating to or involving activities in which people spend time talking to each other or doing enjoyable things with each other.

(https://www.merriam-webster.com/dictionary/social, retrieved 19.10.2021).

Even though this definition is very human centered, social behavior can be found in various species, including other mammals and even insects; for example, the desert locust *Schistocerca gregaria* has the word “social” already in its name, since gregarious is a synonym for social. Not surprisingly, social species like the mentioned desert locust, or rodents like prairie voles or rats are used to study social behavior and the neuroscience of it to get a better understanding of how, when, and why animals are behaving the way they do. Translational studies use animal models to investigate neuropsychiatric disorders with impairments in social behavior, such as autism spectrum disorders or schizophrenia (Jones et al., 2011; Wöhr & Scattoni, 2013). Most of these studies use rodents for these purposes, mainly rats, as rodent and human brain share extensive homology and they show a wide variety of social behaviors (Campbell & Hodos, 1970; Striedter, 2002; Vogt & Paxinos, 2014).

Social behavior in rodents consists of many categories, such as sexual behavior, territorial behavior, and play behavior (Wolff & Sherman, 2007). The function is mainly to maintain social bonds for survival and the fitness of individuals (Wolff & Sherman, 2007). It can contain friendly encounters, like mating and pair bonding during sexual behavior, or unfriendly encounters, e.g. social aggression or defeat during territorial behavior (Lukas & de Jong, 2016). These behaviors can be studied in different paradigms, for example in the social preference test, the social defeat paradigm, or the maternal defense test (Lukas & de Jong, 2016). Most importantly the roles of sender and receiver in social paradigms can be well studied; for example, in playback studies, where USV are presented to test rat’s response towards it on a radial maze (Seffer et al., 2014).

Depending on the presented stimulus material, the response of the subject rat can either induce approach or avoidance behavior.

50-kHz playback paradigm and social approach behavior

Social approach behavior can be best studied during playback paradigms in rats (Wöhr & Schwarting, 2007; Seffer et al., 2014). In this paradigm a rat is placed on an 8-arm radial maze with two ultrasonic speakers and microphones on opposite sides. After an initial habituation phase, playback of USV starts from only one side of the maze. The stimuli can consist of pro-social 50-kHz USV, aversive 22-kHz USV, or time- and amplitude-matched noise as a control stimulus. Furthermore, artificially created tones in the mentioned frequencies can be played back. During playback of pro-social and appetitive 50-kHz USV, the rat approaches the sound source and spends significantly more time in proximity to the active speaker. This response behavior is very strong and reliable and was shown in both male and female rats, as well as in different stocks like Wistar (WI), Sprague-Dawley (SD), and Long Evans rats (Wöhr and Schwarting 2007, 2009, 2012; Burgdorf et al. 2008; Sadananda et al. 2008; Parsana et al. 2012; Snoeren and Ågmo 2014; Willadsen et al. 2014; Willuhn et al. 2014; Seffer et al. 2015; Brenes et al. 2016; Ouda et al. 2016; Pultorak et al. 2016; Saito et al. 2016; Saito and Okanoya 2017; Engelhardt et al. 2017, 2018; Kagawa et al. 2017). Factors such as sex, age, and inter-individual trait-like differences seem to play an important role in the degree of social approach. It has been shown that the social approach is strongest in juvenile male rats, whereas it decreases in adults (Wöhr & Schwarting 2007, 2009; Willuhn et al. 2014; Seffer et al. 2015; Brenes et al., 2016). The reason for the stronger approach towards 50-kHz USV playback is the rough-and-tumble play period during adolescence, where rats learn the social incentives and the positive meaning of 50-kHz USV (Burgdorf et al., 2008). Regarding sex, female adult rats also exhibit a strong social approach (Willadsen et al, 2014). These findings could not be replicated by Snoeren and Ågmo (2014), who presented repeated 50-kHz USV playback, which might have led to habituation. Moreover, not only natural 50-kHz USV stimuli elicited approach behavior but also artificially generated 50-kHz tones were able to induce social approach (Wöhr & Schwarting, 2007). This indicates that frequency modulation is not an important feature for rats to elicit approach behavior, which is also in line with the findings that after separation from a conspecific the animals emit primarily flat 50-kHz USV (Wöhr et al., 2008). This also led to the conclusion that 50-kHz emission is dependent on possible social contact. The 50-kHz USV playback paradigm further makes it possible to analyze the behavior of the receiving rat, rather than the sender of USV representing the reciprocal nature of communication. It can also be used to study communicational deficits in rat models for neuropsychiatric or neurodevelopmental disorders, where communication is impaired (Seffer et al., 2015; Kisko et al., 2018; Berg et al., 2018, 2021a). Besides the social approach behavior towards the sound source, several studies described response calls towards 50-kHz USV playback, which have not been characterized in detail yet (Wöhr & Schwarting, 2007, 2009; Willadsen et al., 2014; Willuhn et al., 2014; Engelhardt et al., 2017, 2018; Berg et al., 2018, 2021a; Kisko et al., 2020; Olszyński et al., 2020; 2021). To this reason, response calls towards 50-kHz USV playback are the core of Study II in this dissertation (Berz et al., 2022).

Habituation phenomenon

Previous studies showed a strong and reliable social approach towards playback of 50-kHz USV is only during the first presentation. A second exposure to the 50-kHz USV playback paradigm was shown to be less effective to induce social approach or even resulting in no approach at all (Wöhr & Schwarting, 2012). This habituation phenomenon towards pro-social USV was shown to be dependent on learning and memory processes by applying systemically the cholinergic antagonist scopolamine after the first playback of 50-kHz USV (Wöhr & Schwarting, 2012). This application prevented memory consolidation of the social acoustic memory of the 50-kHz USV playback and during a second playback, one week later, the rats approached the sound source again; whereas the control group without any treatment did not exhibit social approach behavior (Wöhr & Schwarting, 2012). Additionally, it was shown that the level of dopamine in the NAc decreases when the 50-kHz USV playback is repeated; indicating that the dopaminergic reward system plays a crucial role in social approach behavior (Willuhn et al., 2014).
The quick and sustaining habituation towards 50-kHz USV is not plausible, neither from an evolutionary point of view, nor regarding social behavior and the incentive salience of appetitive 50-kHz USV. To this reason I conducted several experiments to unravel possible reasons for the quick habituation towards playback of 50-kHz USV. Study I in this dissertation describes the details of the phenomenon that include generalizability over different stocks, pharmacological treatment with the dopaminergic agonist d-amphetamine, and the role of the internal state (Berz et al., 2021).

Dopamine and approach behavior

Dopamine (DA) presumably is the most studied catecholamine. The dopaminergic system is one of the core systems of the brain influencing several kinds of behavior (Ikemoto & Panksepp, 1999). Presumably the most important pathways are the mesocortical and the mesolimbic pathways, originating mainly in the ventral tegmental area (VTA) projecting to the striatum and subcortical structures, importantly to the NAc (Gerfen, 2010; Hutson et al., 2014). The nigrostriatal pathway originates in the substantia nigra pars compacta (SNc) and sends afferents to the dorsal regions of the basal ganglia (Zahm & Trimble, 2008). The latter pathway plays a crucial role in movement control, which is why it is a major target in Parkinson's treatment, linked to the degeneration of DA neurons in the SNc (Zahm & Trimble, 2008; Corti et al., 2011). Most of the output regions of the mesocortical and mesolimbic pathways are associated with functions of reinforcement, cognition, and emotional control, which makes the dopaminergic system a suitable target for research on several neuropsychiatric disorders, for example bipolar disorder or drug abuse (Miklowitz & Johnson, 2006; Shen et al., 2012).

Perception and emission of USV have also been associated with the dopaminergic system, especially the perception of 50-kHz USV playback (Sadananda et al., 2008; Willuhn et al., 2014). Moreover, specific brain areas could be linked to 50-kHz USV playback, such as increased activity in the NAc (Sadananda et al., 2008), whereas brain regions associated with fear responses, such as the amygdala, are inhibited (Parsana et al., 2012). These activations are clearly of dopaminergic nature, which was shown in a study investigating phasic dopamine release in the NAc during the 50-kHz USV playback via fast-scan voltammetry (Willuhn et al., 2014). In that study the dopaminergic signaling increased in the NAc during the playback stimuli. With repeated presentation of the 50-kHz USV stimulus, the dopamine response decreased (Willuhn et al., 2014). This indicates that habituation towards repeated playback of 50-kHz USV is depending on dopaminergic activity in the NAc. The production of 50-kHz USV is also clearly linked to the dopaminergic system. Activation of the ascending dopaminergic system from the VTA to the NAc and other forebrain structures of the mesolimbic system leads to increased locomotor activity as well as increasing the emission of 50-kHz USV (Burgdorf et al. 2001; Thompson et al. 2006; Brudzynski, 2007, 2009; Brudzynski, 2013). The activation of this system also indicates a positive affective state (Brudzynski, 2007, 2009, 2013). Therefore, dopaminergic agonists, like amphetamine (AMPH) were chosen to further investigate role of the dopaminergic system in 50-kHz emission (Rippberger et al., 2015; Engelhardt et al., 2017, 2018; Wöhr, 2021). Several studies were able to show that systemic AMPH administration leads to increased social approach during 50-kHz USV playback, as well as elevated emission of response calls following playback (Engelhardt et al., 2017, 2018), which underlines the importance of the mesolimbic dopaminergic system during social approach towards 50-kHz USV playback.

In addition to the increased approach behavior after AMPH administration, treatment with the DA D2 receptor antagonist haloperidol (HALO), leads to a cataleptic state in rats (Tonelli et al., 2017, 2018). This induction of immobility via blocking DA D2 receptors in the striatum, makes it a good model for Parkinson's disease (Johnson et al., 2014; Kharkwal et al., 2016). Playback of 50-kHz USV can induce paradoxical kinesia in rats after HALO-induced catalepsy, which means that the rats "awake" from the cataleptic state during 50-kHz USV playback and are able to approach the sound source. This phenomenon is probably due to DA reserves in the basal ganglia or routes bypassing them (Glickstein & Stein, 1991).

Together, these pharmacological manipulations during 50-kHz USV playback show that the dopaminergic system plays an important role during social approach behavior. Therefore, the pharmacological manipulations in Study I and II were performed with either the DA agonist AMPH, or the DA D2 receptor antagonist HALO to study the underlying neuronal mechanisms of the habituation phenomenon and response calls towards 50-kHz USV playback.

Aim of this Dissertation and research questions

Social approach behavior in rats during appetitive 50-kHz USV playback has been reported to be very strong and reliable across different sexes, ages, and under pharmacological manipulations (Wöhr & Schwarting 2007, 2009, 2012; Burgdorf et al. 2008; Sadananda et al. 2008; Parsana et al. 2012; Snoeren and Ågmo 2014; Willadsen et al. 2014; Willuhn et al. 2014; Seffer et al. 2015; Brenes et al. 2016; Ouda et al. 2016; Pultorak et al. 2016; Saito et al. 2016; Saito and Okanoya 2017; Engelhardt et al. 2017, 2018; Kagawa et al. 2017). However, almost all studies were performed with only one initial playback test. The few research projects that used a repeated playback paradigm indicated that a quick habituation occurs after the first test of 50-kHz USV playback. This habituation was shown to rely on learning and memory mechanisms (Wöhr & Schwarting, 2012). Moreover, most of the prior studies on playback paradigms involved WI rats. Whether the habituation towards 50-kHz USV playback is generalizable over other outbred stocks is therefore not known so far. Even though studies about neuropsychiatric disorders used SD rats (Kisko et al., 2018, 2020) in playback studies, nothing is known about repeated 50-kHz USV playback in them. Additionally, pilot studies prior to the presented studies in this dissertation revealed a possible difference in habituation in the stocks of WI and SD rats. This suggests a comparison of these two stocks in the repeated playback paradigm, which has not been performed yet and would indicate whether the habituation phenomenon is generalizable or not. Furthermore, the quick habituation towards playback of appetitive 50-kHz USV was shown to be coupled with decreases in phasic DA release in the NAc (Willuhn et al., 2014). Other studies proved that systemic treatment with the dopaminergic agonist AMPH can increase social approach behavior towards 50-kHz USV playback (Rippberger et al., 2015; Engelhardt et al., 2017, 2018). Therefore, the question arises, whether AMPH treatment might prevent habituation when applied directly before the retest to increase DA availability.

As already mentioned, habituation relies on learning and memory (Wöhr & Schwarting, 2012) and the retrieval of memories was proposed to be state-dependent (Radulovic et al., 2017). Accordingly, I hypothesized that habituation towards 50-kHz USV playback might be state-dependent as well. To test this, I pharmacologically induced an internal state in the rats with systemic application of either HALO, which was shown to induce a cataleptic state with the remaining ability to approach 50-kHz USV playback (Tonelli et al., 2017, 2018), or saline (SAL). The control groups received the same substance during first test and retest, whereas the experimental groups received HALO or SAL in the first test and the corresponding other substance during the retest to induce a mismatch of internal state between the two tests, to see whether a this can overcome the habituation phenomenon.

Response calls towards 50-kHz USV playback have been reported in several studies (Wöhr & Schwarting, 2007, 2009; Willadsen et al., 2014; Willuhn et al., 2014; Engelhardt et al., 2017, 2018; Berg et al., 2018, 2021a; Kisko et al., 2020; Olszyński et al., 2020; 2021). Nonetheless, no detailed analysis and characterization of these calls has been performed yet. Therefore, I wanted to take the first approach of conducting a profound and detailed characterization of the call features of response calls including call likelihood, temporal patterns, as well as call parameters like mean peak frequency, call duration, and frequency modulation. Response calls had been reported in WI as well as SD rats, but no comparison between the two stocks was conducted to this point. Therefore, I wanted to demonstrate possible generalizability of response calls across rat strains. No neural mechanisms underlying the emission of response calls have been demonstrated yet. Therefore, I analyzed the effect of systemic treatment of the DA D2 receptor antagonist HALO on response calls. Since the emission and reception of 50-kHz USV is based on the dopaminergic system and the 50-kHz USV playback represents an appetitive situation, we hypothesized that response calls during this paradigm might be influenced by the DA system as well.

Research questions

The analyzed and presented data were derived from three behavioral experiments/data sets (Fig. 1). Data regarding the habituation phenomenon included repeated playback of 50-kHz USV, i.e. during a first test and a retest one week later. The results for the response calls were generated in each case only from the initial first test of the same experiments (referred to as data sets). Meaning that the analyzed animals were the same.

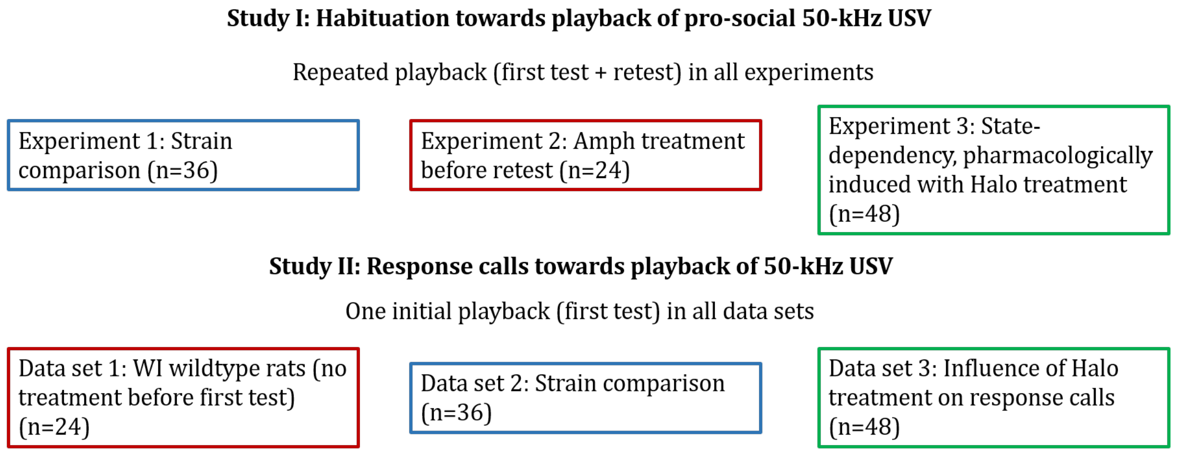

Fig. 1: Experiments/data sets of Study I and II. The same color implies the same experiments and subjects were used and analyzed. Hence, e.g. animals of Experiment 2 in Study I (AMPH treatment before retest) were the same as in Data set 1 of Study II (WI wildtype rats, with no treatment before first test).

Study I: Limited generalizability, pharmacological modulation, and state-dependency of habituation towards pro-social 50-kHz calls in rats.

Is the habituation towards playback of 50-kHz USV generalizable over different stocks?

In a repeated 50-kHz USV playback paradigm, I tested the two commonly used outbred laboratory rat stocks WI and SD rats and compared their behavioral responses.

Does systemic amphetamine treatment override the habituation phenomenon?

Before the retest, the animals received a systemic AMPH injection (i.p., 2.5 mg/kg) and a control group received an injection of the vehicle SAL (i.p., 0.9 % NaCl-solution). The two groups were compared in regard to habituation and behavioral responses.

Is the habituation phenomenon state-dependent?

The internal state of the rats was pharmacologically manipulated with a systemic injection of the dopaminergic antagonist HALO (i.p., 0.5 mg/kg) or the control vehicle SAL (i.p., 0.9 % NaCl-solution). The groups where the internal state differed between the playback trials (SAL-HALO and HALO-SAL) were compared to the groups where the internal state did not change (SAL-SAL and HALO-HALO). The behavioral responses were compared to see whether the change of internal state can prevent the habituation phenomenon.

Study II: Response calls evoked by playback of natural 50-kHz ultrasonic vocalization in rats.

What are the features of response calls towards 50-kHz USV playback and how do they look like?

During an initial playback of 50-kHz USV the calls in response towards the playback was characterized in juvenile male WI rats. The response calls were characterized and compared in regard to number of calls, latency to call after playback onset, and the call parameters mean peak frequency, duration, and frequency modulation.

Do response calls differ between the stocks of WI and SD rats?

During an initial playback of 50-kHz USV the response calls with all call parameters towards playback were compared between the two stocks of WI and SD rats.

Which effect has systemic HALO treatment on response calls?

Again, in an initial playback paradigm the rats received either HALO (i.p., 0.5 mg/kg) or SAL (i.p., 0.9 % NaCl-solution) systemically. The response calls of the two treatment groups were compared in regard to all call parameters.

Summary of publications

Study I: Limited generalizability, pharmacological modulation, and state-dependency of habituation towards pro-social 50-kHz calls in rats.

Berz A, Pasquini de Souza C, Wöhr M, Schwarting RKW (2021). Limited generalizability, pharmacological modulation, and state-dependency of habituation towards pro-social 50-kHz calls in rats. *iScience 24, 102426*.

Journal impact factor: 5.08

Background. Rats as highly social animals use ultrasonic vocalizations (USV) in order to communicate. Calls around 50-kHz are thought to represent a positive affective state and serve as social contact calls (Wöhr, 2018). Studies with playback of natural 50-kHz USV have demonstrated a strong and reliable social approach towards the sound source (Wöhr & Schwarting, 2007; Seffer et al., 2014). However, the social approach behavior was shown to decrease quickly after only one presentation of 50-kHz USV (Wöhr & Schwarting, 2012). This quick habituation was demonstrated to be dependent on learning and memory since it could be prevented pharmacologically with scopolamine, a cholinergic antagonist inducing amnesia (Wöhr & Schwarting, 2012). Furthermore, a decrease of dopaminergic activity in the NAc was shown during repeated playback of 50-kHz USV (Willuhn et al., 2014). This quick and strong habituation is surprising and the reason why such a social signal with high incentive salience is unclear. Therefore, we conducted three experiments aiming at understanding and overcoming the habituation phenomenon in terms of generalizability, pharmacological manipulation, and state-dependency.

Methods. Three experiments with repeated playback of natural 50-kHz on the radial maze were conducted in juvenile male rats. In experiment one, the two rat stocks Wistar (WI) and Sprague-Dawley (SD) rats were compared in regard to their approach behavior towards the sound source in a first test and a retest one week later. Experiment two tested the effect of systemic amphetamine (AMPH) application before the retest compared to a control group receiving a saline injection (SAL) instead. In Experiment three, the internal state of the rats was manipulated pharmacologically with injection of the D2 receptor antagonist haloperidol (HALO). This resulted in four groups with either a change of the internal state (HALO-SAL or SAL-HALO in first test and retest, respectively) or the same internal state between the two playback presentations (HALO-HALO or SAL-SAL). The presented stimuli were natural 50-kHz USV recorded from an adult male rat exploring the cage of a concomitant after it had been removed (for details see Wöhr et al., 2008). As a control stimulus time- and amplitude-matched noise was presented in a counter-balanced manner to ensure that the rats heard the sounds. Approach behavior in the first test and the retest was measured by entries into and time spent on the proximal arms close to the active speaker and the same was done with entries and time on the distal arms opposite the active speaker. For quantification change scores were calculated by subtracting the entries or time on the proximal and distal arms before stimulus presentation from the entries and time spent in the arms during the five minutes of stimulus presentation. Locomotor activity was measured by distance traveled expressed in cm. Additionally, 50-kHz USV were measured and analyzed in experiment two after AMPH treatment in the retest.

Results. We could show that SD rats in comparison to WI rats did not exhibit habituation and approached the 50-kHz USV playback during the retest again. AMPH treatment before the retest led to repeated approach behavior as well and could prevent habituation. A change of the internal state only prevented habituation in the HALO-SAL group. The other groups (HALO-HALO, SAL-SAL, SAL-HALO) did all habituate towards playback of 50-kHz USV. Besides the approach behavior and habituation outcomes, locomotor activity was measured. Locomotor activity during the initial 15 min of the experiment decreased in WI and SD rats, what was more prominent in WI rats, which was also the case in the retest. In the second experiment, AMPH treatment, in comparison to SAL treatment, led to psychomotor activation represented by elevated locomotor activity during the first 15 min. HALO-treatment in experiment three resulted in clearly lower locomotor activity during the first test and the retest. However, during the retest, SAL-HALO animals and HALO-SAL animals exhibited increased locomotor activity compared to their counterparts SAL-SAL or HALO-HALO, respectively. Regarding response calls, animals from the third experiment emitted more response calls towards 50-kHz USV in the first test compared to the retest.

Discussion. The habituation phenomenon seems to be not generalizable, since WI rats display a habituation towards 50-kHz USV playback during the retest, whereas SD rats do not. These stock differences might rely on different aspects. First, it could be the kind of stimulus material and that the 50-kHz USV presented in our experiment have a different informational value. Other subject-dependent factors might play a role, like higher social motivation represented by higher engagement in rough-and-tumble play and increased emission of 50-kHz USV in SD rats (Manduca et al., 2014a, b). Another factor might be the different levels of general locomotor activity. Yet, different studies have shown opposing effects of either more anxious behavior in WI rats compared to SD rats (Rex et al., 2004; Rybnikova et al., 2018), or more novelty-seeking behavior in WI rats, which might explain the elevated locomotor activity during the first 15 min (Walker et al., 2009). Nevertheless, with all the differences between the stocks, it is unlikely that the differences in habituation are driven by factors like anxiety. In the second experiment, AMPH treatment before the retest could prevent habituation, with increased social approach behavior in the treatment group. SAL treated controls did not exhibit social approach during the retest.

However, the two experimental groups did not differ significantly from each other, which might partly be driven by unspecific stimulatory effects in AMPH treated rats. In the third experiment the relevance of the internal state of the animal during first test and retest was analyzed, with systemic treatment of HALO. A mismatch of internal state was shown to hinder memory retrieval (Radulovic et al., 2017). Since the habituation phenomenon is thought to rely on intact memory retrieval (Wöhr & Schwarting, 2012), we wanted to prevent habituation by pharmacologically altering the internal state of the rats between first test and retest. Indeed, rats that had received HALO in the first test and SAL in the retest approached the sound source again. All other groups did habituate, strongly the group that received SAL in the first test and HALO in the retest. This might reflect a floor effect because of the combination of habituation and induced immobility via the drug. Additionally, the rats emitted response calls towards 50-kHz USV playback, which had been reported before (Wöhr & Schwarting, 2007, 2009; Willadsen et al., 2014; Willuhn et al., 2014; Engelhardt et al., 2017, 2018; Berg et al., 2018, 2021a; Kisko et al., 2020; Olszyński et al., 2020; 2021). Importantly, these response calls decreased in all rats of experiment three in the retest compared to the first test.

Together we could show that the habituation phenomenon is of limited generalizability, can be overcome by treatment with AMPH via elevated meso-limbic DA function and relies on intact memory retrieval shown by the prevention of habituation with changing internal states.

Study II: Response calls evoked by playback of natural 50-kHz ultrasonic vocalization in rats.

Berz AC, Wöhr M, Schwarting RKW (2022). Response calls evoked by playback of natural 50-kHz ultrasonic vocalization in rats. *Frontiers in Behavioral Neuroscience*.

Journal impact factor: 3.558

Background. Rats as highly social animals use ultrasonic vocalizations (USV) for communication. 50-kHz USV are believed to represent a positive affective state and function as social contact calls (Wöhr, 2018) whereas 22 kHz calls are believed to serve as alarm or distress calls (Brudzynski, 2021). Playback studies of natural 50-kHz USV have demonstrated a strong and reliable social approach towards the sound source (Wöhr & Schwarting, 2007; Seffer et al., 2014). Besides the approach behavior, no other measurements have been done so far during 50-kHz USV playback to prove a possible reciprocity of the receiver rat. We tried to close this gap by showing and characterizing USV emitted by the receiver rat during an initial 50-kHz USV playback paradigm. Such response calls had been reported before in male and female rats (Berg et al., 2018, 2021a) with factors including age (Wöhr & Schwarting, 2009), prior experiences (Olszynski et al., 2021) and inter-individual differences (Engelhardt et al., 2018) playing an important role. However, the detailed call parameters and function of response calls remain unknown, which is why we analyzed response calls and compared rat stocks, namely WI and SD, and investigated whether pharmacological treatment with the dopaminergic antagonist haloperidol affects response calls towards 50-kHz USV playback.

Methods. We capitalized on a previously obtained large data set of 50-kHz USV playback, where the behavioral results of the approach behavior had already been published (s. Study I: Berz et al., 2021). We divided the data into three data sets. Data set 1 included 24 WI rats to test whether response calls are specific to 50-kHz USV and not noise and whether the emission depends on stimulus order. The second data set consisted of 18 WI and 18 SD rats to compare the emission of response calls in these stocks. The third data set was comprised of 24 rats receiving the dopaminergic D2 receptor antagonist haloperidol (HALO) and 24 control rats receiving saline (SAL). The USV data were analyzed with DeepSqueak, a deep learning-based system for detection and analysis of USV (Coffey et al., 2019). After denoising with a custom-trained network, the files were transferred to the DeepSqueak Screener (L. Lara-Valderrábano and R. Ciszek: 10.5281/zenodo.3690137[1]) where they were manually accepted (response calls) or rejected (noise or playback calls). The call parameters mean peak frequency, call duration, and frequency modulation were analyzed.

Results. Most rats emitted response calls towards playback, specifically towards 50-kHz USV but not noise. Emission of response calls started immediately after 50-kHz USV was played and all rats exhibited a strong social approach (s. Berz et al., 2021). The majority of the calls was below frequencies of 32 kHz, the threshold between 22- and 50-kHz calls, and with a duration around 0.3 s and frequency modulations below 5 kHz. These parameter values correspond to short 22-kHz USV rather than 50-kHz USV. Despite the large variability among calls, WI and SD rats emitted response calls without compelling differences regarding numbers of emitted calls, latencies to start calling, and call likelihood. Additionally, their mean peak frequency, call durations, and frequency modulations did not yield significant differences. However, SD rats clearly showed calls around 50-kHz, which was not as prominent in WI rats. Treatment with the D2 receptor antagonist HALO did not show a significant effect on response calls. SAL-treated rats as well as HALO-treated rats exhibited similar call rates, latencies, temporal distribution, peak frequency, and frequency modulation. The only difference was that HALO-treated rats had significantly longer call durations than SAL-treated rats.

Discussion. We showed that response calls towards playback of natural 50-kHz USV is a robust phenomenon among WI and SD rats and that they are not affected by treatment with the

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[1] https://github.com/UEFepilepsyAIVI/DeepSqueak.git

dopaminergic antagonist HALO. The call parameters resemble those of short 22-kHz USV, which are thought to represent a dysphoric state or displeasure without any external threat (Simmons et al., 2018). This leads to the assumption that response calls might be an expression of frustration after occurring expectation violation. In line with this hypothesis is the fact that most animals started calling with higher frequencies, which quickly changed to calls in the 22 kHz range. Another hypothesis for the function of response calls might be an ambivalent emotional state. Beside the fact that the two affective states, namely appetitive (50-kHz USV) and aversive (22-kHz USV), have been proposed to be mutually exclusive (Brudzynski, 2021) they might oscillate quickly between each other. This is supported by the emission of short 22-kHz calls and 50-kHz calls, especially in SD rats. The final hypothesis about the function of response calls might be for appeasing purposes. In adult male rats USVs of lower frequencies were found during play fighting in situations at risk to escalate into aggression (Burke et al., 2017a, 2020). The call parameters in Burke et al. (2017a) were very similar to the reported frequencies, durations, and frequency modulations in our study. Furthermore, the playback in our study was obtained from an older rat and presented to juvenile subjects. So far, it is not known whether rats can gain information about the age of a calling rat, but the emission of response calls in the receiving rats might be to appease an assumed older concomitant. Therefore, response calls might serve as social contact calls as their emission was primarily during 50-kHz USV playback and accompanying social approach behavior in order to establish contact. Taken together, the response call phenomenon studied here in detail appears sufficiently robust to be used as a measure for the reciprocal nature of acoustic communication and can easily be applied in rat model systems for neuropsychiatric disorders, where acoustic communication is impaired, such as autism spectrum disorder (Lai & Baron-Cohen, 2015). This offers a new approach to studying the reciprocal nature of communication in rodent models for neuropsychiatric disorders.

Discussion

Albeit the habituation phenomenon and that it is based on learning and memory (Wöhr & Schwarting, 2012) has been known and studied before, no detailed analyses of how to overcome this phenomenon have been conducted to this point. Therefore, this dissertation project aims at providing insight into methods and mechanisms on how to overcome the habituation phenomenon. Further, it indicates which role response calls towards 50-kHz USV playback hold.

I performed a series of experiments to study the reasons and mechanisms of the habituation phenomenon (Study I). The results showed that the phenomenon does not apply to all rat stocks and hence is not generalizable. Only WI rats but not SD rats habituated towards playback of 50-kHz USV in the repeated playback paradigm. Nonetheless, pharmacological manipulations proved to be effective in regard to the habituation phenomenon. Treatment with the dopaminergic agonist AMPH directly before the retest prevented habituation towards 50-kHz USV playback. Pharmacological manipulation of the internal state of the animals with the DA D2 receptor antagonist HALO showed that the habituation phenomenon is state-dependent. If the internal state of the rat changes between first test and retest the habituation was inhibited as well. This was particularly the case for animals receiving HALO in the first test and SAL in the retest, possibly representing an additive effect of the immobility and hence lack of exploration in the first test and the valence of the 50-kHz USV playback. Various factors playing a role during habituation are discussed in the following paragraphs to further unravel this phenomenon.

Response calls towards 50-kHz USV playback have been reported in previous studies (Wöhr & Schwarting, 2007, 2009; Willadsen et al., 2014; Willuhn et al., 2014; Engelhardt et al., 2017, 2018; Berg et al., 2018, 2021a; Kisko et al., 2020; Olszyński et al., 2020; 2021), however, their characteristics or functions have not been described in detail yet. To this reason I capitalized on the data obtained in Study I and analyzed the USVs arising in the first playback test of the three conducted experiments (Study II). I showed that WI as well as SD rats emit response calls towards 50-kHz USV playback and that a pharmacological treatment with HALO did not affect emission of these calls. Three different theories for the meaning and function of response calls were proposed. The first hypothesis was about the ambivalent state the animals experience during playback of 50-kHz USV. The two different affective states, appetitive and aversive, were suggested to be mutually exclusive (Brudzynski, 2021), where the rats emit either appetitive 50-kHz or aversive 22-kHz calls, respectively. Since the response call parameters are similar to those of 22-kHz calls, the animals were supposedly in a negative affective state. SD rats, however, also emitted calls around 50-kHz, which clearly speaks against the hypothesis of a solely aversive affective state. It is possible that the states change quickly between each other in an oscillating manner representing an ambivalent state. Another hypothesis about the function of response calls was their possible appeasing nature. Other studies showed that older rats emit 50-kHz calls but also calls around 30 kHz during play fighting, especially in situations that are at risk to escalate (Burke et al., 2017a, 2020). The response calls in Study II resemble these calls. So far, it is not known whether rats can gain information about the age via USVs. Since the calls in Study II used as playback stimuli were obtained from an older rat presented to juveniles, it might be that the subjects wanted to appease the assumed concomitant. The last hypothesis concerns the possible frustrated state the rats might experience during 50-kHz USV playback by hearing the calls but not being able to approach and interact the expected playmate. Several situations where response calls or calls of lower frequencies occur will be discussed in this section.

Various factors playing a role during habituation

Factors playing a role during habituation might be of sexual nature, depending on age, or the type of stimulus material could be important. Furthermore, neural correlates could explain the habituation phenomenon and additional experiments indicate that a social consequence after the first test is not preventing habituation.

In Study I, only juvenile male rats were used, which are mostly used in research. It is also shown that adult and juvenile female rats show a reliable strong social approach towards 50-kHz USV playback originating from a male, indicating no significant difference in sex (Willadsen et al., 2014; Berg et al., 2020). However, also contradictory findings argue that male 50-kHz USV do not have incentive value to adult females (Snoeren & Ågmo, 2014). The animals could have habituated towards the playback due to the repeated 50-kHz USV in the study by Snoeren & Ågmo. The authors nevertheless conclude, that there was no difference in approach behavior between the test days, speaking against a habituation.

Further unpublished data showed that adult female rats do not show habituation towards 50-kHz USV playback. In a pilot study prior Study I and II in this dissertation, I tested adult female wildtype rats and rats haploinsufficient for the *Cacna1c* gene and the results indicated, that the social approach behavior was the same during first test and retest. This might be an indication that male USV does have an incentive value for female rats, even more than for male rats, opposing the conclusion by Snoeren & Ågmo.

Another factor that might determine habituation could be the type of stimulus material used for playback on the radial maze, which was always the same sequence of calls. These calls were recorded from an adult male rat exploring a cage with scents of a strange conspecific after it was removed from the cage (Wöhr et al., 2008). The quick habituation towards these particular 50-kHz USV suggest a decrease in incentive valence for the receiver. However, speaking against the fast decline of incentive valence is that playback studies of other USV stimuli, like pup USV, towards their mothers did not induce habituation. On the contrary, when mothers were separated from their pups and pup USV were played back, this induced repeatedly retrieval behavior of the mother and returning her pups back to the nest (Smith, 1976; Ehret & Haack, 1981, 1982; Ehret, 1992). This is an indication that different types of USV have different incentive valence. Therefore, it would be interesting to test other 50-kHz USV sequences during the retest of the repeated playback paradigm.

Neural correlates underlying the habituation phenomenon are also very important to understand this phenomenon. It was shown that habituation, or rather social acoustic memory, is based on the cholinergic system, which is important for learning and memory. This was demonstrated in the repeated playback paradigm with systemic treatment of the muscarinic acetylcholine antagonist scopolamine (known to induce amnesia) directly after the first test (Wöhr & Schwarting, 2012). Another lesion study showed that the basolateral amygdala (BLA) seems to play a role in social approach behavior (Schönfeld et al., 2020). They tested the rats in three consecutive sessions and social approach behavior was significantly higher during the first playback compared to the following one. This decrease in approach behavior represents a habituation and even though the BLA lesioned animals showed attenuated approach behavior during the first test, they also habituated over time. This indicates that the BLA is involved in certain behaviors towards salient stimuli, but not habituation towards them (Schönfeld et al., 2020).

Furthermore, preliminary results of an experiment with a social consequence after the first 50-kHz USV playback indicate that the anterior cingulate cortex might play a role in residual approach behavior during the retest. During this additional experiment about habituation towards 50-kHz USV playback, the question whether habituation occurs because of a missing play partner to associate with the 50-kHz playback was addressed. Therefore, I performed an adjusted 50-kHz USV playback paradigm with a social consequence after the 50-kHz playback in the first test (unpublished data) to further unravel the question, why rats habituate.

In this experiment, the paradigm used in Study I was adapted, so that all subject rats were exposed to one 50-kHz USV playback after the 15 min habituation on the radial maze. This concluded in a paradigm of 20 min duration, rather than the 45 min in experiments in Study I, because no time- and amplitude-matched noise as control was presented. A counter balanced paradigm with both stimuli would not have been possible, because directly after the 50-kHz USV playback, the rats were taken off the maze. Directly after hearing 50-kHz USV playback, they underwent one of three different conditions: (1) they were either put together with a non-familiar play partner in a separate room, (2) they were put back into their home group cage, (3) rats were put in a single cage in another room. In case of putting the test subject into a cage in another room, they were kept there for 10 min either by themselves or together with a play partner before they were put back into the home group cage. One week later, the retest was conducted under the same conditions to see whether a social consequence after the first test had an influence on approach behavior in the retest. Surprisingly, the preliminary results were exactly the opposite of the hypothesis. All animals, regardless consequence after the first test, did habituate. Habituation was even strongest in rats that had experienced a social consequence and played with an unfamiliar partner, whereas "control" animals that were put back into their home group cage showed the least habituation with a mild social approach towards 50-kHz playback. Several factors could be the reason, why a social consequence did not lead to prevention of habituation. First of all, the social consequence did not take place on the radial maze but in a separate room. Even though the transfer from the radial maze was carried out immediately after the 50-kHz USV playback presentation and was done very carefully in a new cage by an experienced experimenter, this might have led to stress in the animal and therefore the association of the playback and the play partner could not be formed. Additionally, the conditions in the other room where the animals were kept for their play session could have been disturbing. Even though it was dimmed and no bright white light, this could have upset the tested animal, because they are nocturnal animals and are most comfortable in the dark (or red light as on the maze, since they do not possess receptors for red). Furthermore, meeting a new play partner could have increased stress for the animals. Previous studies have shown that strange juvenile partners engage vividly in rough-and-tumble play when put together. In the described study, the unfamiliar rat had also been a juvenile male rat with the same size and age and play was also observed between the pairs. Nevertheless, some of the mentioned factors might have been not rewarding for the test subject, which is why no association between appetitive 50-kHz playback on the maze and the following play partner could be formed. Neural correlates measured after the repeated playback indicated that the anterior cingulate cortex plays a role in residual approach towards 50-kHz USV playback in the retest. On an individual level rather than a group level I found a positive correlation of active cells in the anterior cingulate cortex and the approach behavior exhibited during the retest. Even though this experiment does not completely explain why or why not animals habituate regardless social consequence, indicators for neural mechanisms underlying repeated social approach were found.

Possible reasons for the emission of response calls

Are response calls representing an appetitive, ambivalent, or aversive state, or are they just dependent on age and experience? These questions are discussed below to look at the emission of response calls in lower frequency ranges from different perspectives besides the 50-kHz USV playback paradigm.

Over the lifespan of a rat, USV change regarding frequencies and durations. In an aversive foot-shock paradigm, Boulanger-Bertolus et al. (2017) found that juvenile rats seem to emit calls of higher frequencies and shorter durations, whereas adult animals emitted longer calls of lower frequencies (Blumberg et al., 2000; Boulanger-Bertolus et al., 2017). In addition, other studies showed experience-based emission of 22-kHz USV as well as 50-kHz USV in rats and that the association of 22-kHz USV with aversive situations has to be learned (e.g., Endres et al., 2007; Bang et al., 2008; Wöhr & Schwarting, 2010). Such situations can be for example of sexual nature. In studies where predominantly sexually naïve males encountered estrus females it was shown that the males produced calls that had a 22-kHz and a 50-kHz USV component within one breath (Hernandez et al., 2017). These kinds of calls were similar to calls in lower frequency ranges during post-ejaculatory phases when the male was retracting from the female or in a non-contact test where they could see, hear and smell a female but were separated from it with a Plexiglas barrier. In those studies, different call classes in lower frequency ranges were identified representing possibly either a relaxed or a frustrated emotional state of the male rat since they could not reach the females (Bialy et al., 2019). Such short 22-kHz calls were also suggested to represent a dysphoric state or displeasure without any external threat (Simmons et al., 2018). The response calls' parameters in Study II were similar to the ones of short 22-kHz calls, which suggests that the animals in Study II might have been in an aversive or dysphoric state, i.e. frustration. This is in line with other studies also suggesting that the emission of response calls could represent a frustrated state during 50-kHz USV playback due to not being able to reach the possible play partner indicated by the playback (Wöhr & Schwarting, 2009). Frustration is defined as a result of a non-available expected reward after motivational behavior (Burokas, et al., 2012; Scull et al., 1970). In the described playback paradigm of Study II, when the rat realized that there was no rat physically present for interaction after hearing the 50-kHz USV playback, this could have led to a state of frustration in the approaching rat. This might be due to the rat's expectation during 50-kHz USV playback of another rat being present is violated and the rat is not able to reach the potential conspecific. Nevertheless, the additional experiment described above with a social consequence after the first 50-kHz USV playback could not answer this hypothesis completely.

Besides the described aversive or frustrated state, the rats might have been in an ambivalent state during the otherwise appetitive 50-kHz USV playback. As mentioned, the emission of calls below 32 kHz indicates that the rat is in a negative affective state. On the other hand, especially SD rats in Study II emitted 50-kHz USV implicating a positive affective state. Even though these two states have proposed to be mutually exclusive acting in an antagonistic manner (Brudzynski, 2021), it might be that the two different affective states oscillate quickly between each other during the playback.

Apart from an aversive or ambivalent state, studies have also shown low frequency calls in addition to 50-kHz calls during neutral situations, or even during appetitive situations, such as tickling (Barker et al., 2010; Robakiewisz et al., 2019). Therefore, the hypothesis arises that response calls might be of appetitive nature and serve as contact calls in order to remain or establish social contact with conspecifics (Wöhr et al., 2016; Wöhr, 2018). It was shown that mainly flat 50-kHz calls and not frequency modulated calls function as social contact calls (Wöhr, 2018). In experiments where rats were separated from conspecifics, they mainly emitted flat 50-kHz USV probably in order to re-establish contact (Wöhr et al., 2008). In favor of this hypothesis is that response calls in Study II are emitted during social approach behavior and contain mainly flat frequency components.

In line with this hypothesis is that similar calls of lower frequencies and shorter durations have been found during play fighting in adult male rats, another usually appetitive paradigm (Burke et al., 2017a, 2020). In situations that were at risk to escalate these calls were emitted to de-escalate the situation without emerging into aggression (Burke et al., 2017a). It has not been proven that receiving rats can gain information about the age of the USV sending rat. Nevertheless, a previous study found that rats can discriminate between 50-kHz USV from a familiar or a strange rat and that they prefer USV from a strange rat shown by increased self-administration via lever pressing (Vielle et al., 2021). Whether this is also the case for age is not known yet. However, if so, this would support the hypothesis that the receiving juvenile rat during the 50-kHz USV playback of an adult strange rat recognized it as that and wanted to appease the potential opponent in order to establish social contact.

Taken together, most USV of lower frequencies and short durations were mainly found during aversive situations. However, Study II is one of the rare studies to find low frequency calls in an appetitive situation of 50-kHz USV playback. This indicates that the function of the commonly known categories of USV, namely aversive and appetitive, might not be as strict as previously assumed. Even though more research needs to be conducted to investigate the precise meaning of response calls, Study II is a good approach towards analysis of reciprocal communication and raises new opportunities to not only study the receiver's approach response during playback, but also turning the receiver into a sender simultaneously.

Habituation and response calls

As already mentioned, response calls towards 50-kHz USV playback have been reported in several studies (Wöhr & Schwarting, 2007, 2009; Willadsen et al., 2014; Willuhn et al., 2014; Engelhardt et al., 2017, 2018; Berg et al., 2018, 2021a; Kisko et al., 2020; Olszyński et al., 2020; 2021). These studies were all based on one initial playback test and not a repeated paradigm, which investigates the habituation phenomenon. One part of this dissertation aimed at unraveling the mechanisms of habituation towards 50-kHz USV playback (Study I) and the other part characterized response calls towards 50-kHz USV playback in a first playback test (Study II). The remaining question is obvious: Is the habituation phenomenon represented in the emission of response calls? The hypothesis regarding this question is that with habituation towards 50-kHz USV playback from first test to retest, the emission of response calls decreases, whereas during repeated approach behavior the emission of response calls remains high. A first attempt to answer this question was done in the experiment of state-dependency in Study I. All treatment groups, SAL or HALO during the first test emitted response calls towards the 50-kHz USV playback, and during the retest all groups showed a decreased emission of response calls towards 50-kHz USV. This was represented by the significant Chi^2 test results in all experimental groups (for details see table S1 in the appendix). Based on these results from Study I, I conducted additional analyses with the Chi^2 test with the other experimental groups of Study II. The hypothesis of decreased response calls accompanying habituation behavior in the retest of Study I, could only be verified partly. In the experiment of stock comparison, WI rats habituated towards 50-kHz USV playback, which was also represented in decreasing emission of response calls from first test to retest (details in table S1 in the appendix). Regarding SD rats, the hypothesis could also be validated. SD rats did not habituate towards 50-kHz USV playback and as well their emission of response calls remained high during the retest. Considering the experiment of AMPH treatment, the hypothesis had to be rejected. Even though SAL-treated rats habituated and AMPH-treated rats did not, the emission of response calls decreased significantly in both treatment groups from first test to retest. The last experiment and last data set about internal state induced pharmacologically with HALO showed a significant decrease in response calls in all treatment groups but only the group HALO-SAL did not habituate, whereas the three other treatment groups habituated towards 50-kHz playback, as already mentioned above. This is a first indication that habituation towards 50-kHz USV playback might be represented not only on a level of social approach but also by the emission of response calls.

Several aspects could be the reason for rejecting the hypothesis of less response calls in the retest when habituation takes place. First of all, it is well known that some animals emit more USV, whereas other animals do not call at all (callers versus non-callers). This has been shown in several situations and experiments but the reasons behind this phenomenon could not be explained entirely yet (Burgdorf et al., 2005; Mällo et al., 2007). Hence, during the paradigm of repeated 50-kHz USV playback and simultaneous response calls towards playback, the probability of animals approaching the sound source is much higher than animals emitting response calls. The social approach behavior was shown to be strongly reliable, regardless sex, age, pharmacological treatment or other factors like housing conditions (Willadsen et al., 2014; Seffer et al., 2014; Berg et al., 2018; Braun et al., 2019). However, USV emission is not as reliable, regardless the conditions. In the experiments of Study I, not all animals emitted response calls, not even during the first test, but all animals exhibited a strong social approach. Therefore, the decreased number of animals emitting response calls in the retest is a first indicator that habituation could be shown not only on the level of approach behavior but also on the level of response call emission. Nevertheless, more research needs to be done to investigate the relation between response call emission and habituation towards 50-kHz USV playback.

Role of the dopaminergic system

Numerous studies showed that during 50-kHz USV playback the dopaminergic system plays an important role in regard to social approach behavior. The emission of 50-kHz USV was shown to depend on the dopaminergic system (Willuhn et al., 2014) and in the case of social approach towards 50-kHz USV playback, treatment with the DA agonist AMPH increases approach behavior (Rippberger et al., 2015; Engelhardt et al., 2017, 2018). Therefore, in Study I the pharmacological manipulations were performed with the DA agonist AMPH, to prove whether it can prevent habituation. Pharmacologically inducing an internal state and a mismatch of this between first test and retest was done via the DA D2 receptor antagonist HALO. Study I showed that AMPH treatment before the retest of the repeated 50-kHz USV playback can inhibit habituation via elevated meso-limbic DA function and the animals approached the sound sources again. During treatment with HALO inducing a mismatch of internal state between first test and retest results showed that the change of internal state can override habituation.

Regarding response calls towards playback of 50-kHz USV playback, no neural mechanisms underlying their emission are known yet. Systemic treatment with the DA D2 receptor antagonist HALO should shed light onto the question whether response call emission is dependent on the dopaminergic system, since 50-kHz USV emission is depending on this neurotransmitter system. The results of Study II showed that response call emission still occurs and no changes in call likelihood, temporal patterns or call parameters were obvious. Whether response call emission is independent of the entire dopaminergic system cannot be stated exactly, but the DA D2 receptor is definitely not involved in the emission of response calls towards 50-kHz USV playback.

Expectations and expectation violation in the playback paradigm

> Expectation, noun, /ˌɛkˌspɛkˈteɪʃ(ə)n/:
>
> The action or fact of anticipating or foreseeing something; the belief that something will happen or be the case.
>
> *Lexico by Oxford; retrieved 04.11.2021*

Expectation in a general view are beliefs about what might happen in the future (Roese & Sherman, 2007; Hoorens, 2012). Expectations, or expectancies, are a core mechanism of psychology and survival in humans as well as animals referring to collecting information about certain circumstances or situations and transferring it to following behavior (Roese & Sherman, 2007). In humans, expectancies are also important features of mental disorders, e.g. in general anxiety disorder where negative expectations about certain situations persist (Rief et al., 2015). When expectancies are violated, different cognitive responses have been identified: accommodation or immunization. In case of accommodation the expectations are updated after the initial ones were violated, hence in a subsequent similar situation the subject will have changed and adjusted their beliefs about what will happen according to what happened last time. During immunization the beliefs and expectations persist, even though they had been violated. To investigate maintenance and violation of expectations, the research training group 2271 "Breaking expectations" of the University of Marburg has developed a structured and advanced model: the ViolEx model 2.0 (short: ViolEx 2.0; Panitz et al., 2021). This model enables to apply it to many different subfields of psychology, not only, for example, clinical psychology, personality and differential psychology, but also behavioral neuroscience. To communicate and incorporate all the different research fields and findings about expectations and expectation violation three levels of the ViolEx 2.0 model have been developed.

In the case of animal behavior research, and especially the 50-kHz USV playback paradigm in rats, the ViolEx 2.0 level 1 and, to some extent, level 2 are the most convenient ones. In the level 1 of the model, a generalized expectation is applied to a situation specific expectation and can lead to an anticipatory reaction that influences the situational outcome and when the expectation is violated, it leads to either accommodation or immunization. Depending on the cognitive response, the generalized expectation is updated (accommodation) or persists (immunization). In the 50-kHz USV playback paradigm, we can assume that the rat expects something (a conspecific or similar) close to the speaker where the calls are coming from. The anticipatory reaction is then represented in the reliable approach behavior (Wöhr & Schwarting, 2007; Seffer et al., 2014). When the rat realizes that there is nothing but a speaker as the sound source, the expectations are violated. Extending this mechanism to the repeated playback, I found that the rat seems to accommodate the expectations. During a second playback one week later, the rat will not approach the sound source anymore and hence habituates, representing the updated generalized expectation.

In general, the read-out in animal experiments is of observable nature since the animal cannot be questioned about their internal state, which is why only some parts of the ViolEx 2.0 Level 2 can be applied to the repeated 50-kHz USV playback; for example, the part of the external anticipatory reaction with either approach or avoidance. In the 50-kHz USV playback paradigm, this is clearly represented by the strong approach behavior towards 50-kHz USV playback of the receiving rat representing the external anticipatory reaction. Subsequently, this external anticipatory reaction influences the internal representation of the situation and leads to a situation-specific expectation of the rat in this specific paradigm. The following violation of the expectation is apparently of greater extent since the rat will show a response of accommodation, which leads to the quick habituation towards 50-kHz USV playback.

The social and personal influences play a crucial role in the ViolEx 2.0, especially in the Level 2 of the model. These differences influencing the response towards expectation violation can be found in experiment 1 of Study I here. By comparing different rat stocks, the individual differences as well as socio-cultural context, if that can be applied in a rat model, became obvious. WI rats accommodated the behavior after expectation violation in the first playback test and habituated in the retest by not approaching the sound source anymore (approach changed to avoidance). Whereas SD rats did not exhibit such a habituation, therefore did not accommodate and did not change their external anticipatory reaction. In a wider sense, this might depict the “socio-cultural context” as the reason for differences between the rat stocks. Although one has to be very careful to not anthropomorphize animals, it has also been shown that there are differences between stocks, which, however, are often referred to as differences in genetic background and breeding (Schwarting, 2018a, b). Hence, the “socio-cultural” context could be applied in a broader sense.

Moreover, the current state that is depicted in the ViolEx 2.0 Level 2 states the social and personal influences that can affect the generalized expectation (Panitz et al., 2021). This can also alter the external anticipatory reaction, more precisely the approach or avoidance behavior. Manipulating the current, or internal, state is demonstrated here in experiment 3 of Study I. Via pharmacological manipulation with the D2 receptor antagonist HALO and differing internal states between first test and retest of the repeated 50-kHz USV playback, the rat did not exhibit habituation and approached the sound source again; hence, the rat did not accommodate and the anticipatory reaction persisted and was not updated.

In Study II the hypothesis was stated that the quick habituation or accommodation after expectation violation in the first test of the repeated playback paradigm might be due to frustration. Frustration is evoked by the absence of an expected reward (Negrelli et al., 2020). Clearly this reward and t therefore the expectation is violated and leads to a frustrated state in the receiving rat. Importantly, this frustration is represented by the emission of response calls in lower frequencies, usually emitted during a negative affective state (Simmon et al., 2018). With the characterization of these response calls towards playback of natural 50-kHz USV, it was possible to provide further indications for the frustration hypothesis already mentioned in earlier studies (Wöhr & Schwarting, 2009). Therefore, it can be stated that the violated expectation in the playback paradigm evokes a quick response in the receiving rat represented by the emission of response calls, which serve as an expression of a frustrated state (Berz et al, 2022).

Overall, the ViolEx model provides a framework to apply the topic of expectation and violation of expectations in animal models and behavioral paradigms such as the playback paradigm in rats. Many aspects of the model can be applied to explain the phenomenon of changing or maintaining expectations, such as the social and personal influences, especially the current state and the socio-cultural context, the external anticipatory reaction, or the mechanism of accommodation after expectation violation.

Limitations

> "[...] The thoughtful and honest observer is always learning more and more of his limitations; he sees that the further knowledge spreads, the more numerous are the problems that make their appearance."
>
> -Johann Wolfgang von Goethe (1906) from Saunders et al., 1908-

Every great work has its limitations upon one can build on. Also, the studies presented in this dissertation. In the following, some of these limiting factors are discussed in order to better understand and interpret the results of the studies.

Sex and age. In basic research, it is widely common to only use one sex. Yet, this bears many disadvantages because the results are not representative or translatable to humans. We specifically chose male rats because it is believed that they are not under a great hormonal influence, as females are, even though there are already studies proving that this is not the case (Zucker & Beery, 2010). Furthermore, when using both sexes it would have been necessary to double the number of animals in order to have groups big enough for statistical measures. This would not be compliant with the 3R principle (refine, reduce, replace) that is the common rule in animal experiments. Especially it would have been against the second term of reducing animals; instead of reducing the animal numbers, it would have doubled the numbers. It would be interesting to further investigate the phenomena of habituation towards 50-kHz USV playback and response calls evoked by 50-kHz USV playback in both sexes. Especially after Berg et al. (2018) showed that male rats with a deletion of the *Shank3* gene emit more response calls compared to females. This would be interesting to investigate in other stocks without genetic manipulation as well. In both studies only juvenile rats were used. This is common in studies of social approach behavior, since juveniles are more sensitive to USV during the phase of rough-and-tumble play (Burgdorf et al., 2008). As indicated by unpublished data, also adult rats show a habituation and emit response calls. However, these response calls towards 50-kHz USV playback looked different to the ones described in Study II. This would be another interesting fact to look further into.

Power of the statistical tests. Before the experiments were started, a power analysis to calculate the minimum animal number for each experimental and control group was performed. Most of the results reported in Study I and II are significant. Nonetheless, further experiments with larger groups would be good to replicate and validate the results, "ideally including aggregated preference measures for comparing experimental conditions" (Berz et al., 2022).

Pharmacology. Even though it was shown that the unspecific locomotor activity of AMPH treatment and the increased social approach towards appetitive 50-kHz USV playback are different mechanisms (Wöhr, 2021), other catecholaminergic agents could be tested in the repeated playback to verify the involvement of the dopaminergic system being responsible for preventing habituation. Additionally, the effects of the DA D2 receptor antagonist HALO were tested to pharmacologically induce an internal state and to see whether a change in internal state can inhibit the habituation. The results were not as clear as in the other experiments proving prevention of habituation. Due to the induction of immobility by HALO it could be an additive floor effect with habituation and HALO-induced immobility during the retest for animals that received HALO before the retest. Therefore, other catecholaminergic antagonist could be tested, that might not induce immobility, but change the internal state to prove the specificity of the change of internal state preventing habituation.

Additionally, AMPH was only applied before the retest, which was subject of Study I. In Study II, only response calls during the first test were analyzed. Further analysis could focus on pharmacological studies on response calls, as already stated in Study II. Here, the effect of the dopaminergic antagonist HALO on response calls was analyzed. This gave rise to the conclusion that response calls towards 50-kHz USV playback are not dependent on the DA D2 receptor, since the emission took place after HALO treatment.

Anxiogenic features. The radial maze holds possible anxiogenic features due to its elevation from the floor, like it was shown for the elevated plus-maze (Rex et al., 2004; Rybnikova et al., 2018). However, several studies have shown that the elevated radial maze is not anxiogenic since the animals exhibit natural exploration behavior and show a reliable social approach behavior (Wöhr & Schwarting, 2007, 2009, 2012; Willadsen et al., 2014; Seffer et al., 2014, 2015; Rippberger et al., 2015; Engelhardt et al., 2017, 2018). Nevertheless, further experiments such as the open field or cage tests could be conducted to test whether habituation occurs in less anxiogenic environments.

Generalizability. In both studies only the two rat stocks WI and SD rats were tested. To prove generalizability, more stocks should be tested to see whether there are differences regarding habituation or response call emission. Even though habituation towards 50-kHz USV playback was also seen in Long Evans rats and even mice (Hammerschmidt et al., 2009; Schönfeld et al., 2020), response calls were only reported for WI and SD rats. Therefore, the emission of response calls should be analyzed in different rat stocks such as Long Evans rats.

Stimuli. The presented 50-kHz USV stimuli in both studies and all other studies reporting response calls were the same. Albeit the composition of the stimulus sequence represents a wide variety of subcategories of 50-kHz calls (trills, step, flat, mixed; see Wöhr et al., 2008; Kisko et al., 2018), we do not know which valence the USV sequence has for the rats. Previous studies showed that also artificial sine-wave tones with frequencies and durations of 50-kHz calls, but without frequency modulation could induce approach behavior and evoke response calls. Yet, this was only tested during an initial playback and the response calls were not characterized in detail (Wöhr & Schwarting, 2007). Therefore, it would be beneficial to test other 50-kHz USV sequences to see the effect on habituation in the repeated playback paradigm and further characterize response calls towards the different stimuli.

Application and future perspectives

Communication via USV in rodents has long been a great tool to investigate affective states in variable different situations in order to research why the animals are behaving how they do. Not only the sending animal (sender) and its possible affective state can be studied in several social or baseline experiments like the open field test or during tickling. Additionally, the receiver's behavioral response can be analyzed via paradigms like the USV playback. This dissertation elegantly combines the investigation of sender and receiver of USV. During the repeated playback paradigm, the behavioral response of the receiver is analyzed. Because the receiver itself emits response calls towards 50-kHz USV playback, it simultaneously becomes the sender. This not only shows the reciprocal nature of communication, but also the great potential of the combination of USV playback and measuring response calls. It opens new doors for analyzing, for example, several neuropsychiatric disorders, where communication is impaired like autism spectrum disorder (ASD), the Angelman Syndrome (AS) or schizophrenia.

ASD is accompanied by communication and social behavior impaired in humans (American Psychiatric Association, 2013). Thus, USV research in rodents serves as a good method to investigate communicative and social impairments in ASD (for review see Wöhr & Scattoni, 2013). The three relevant core symptoms of ASD are social deficits, impairments of communication, and repetitive behaviors (American Psychiatric Association, 2013). The cause of ASD has not been fully discovered yet but many indications are given that it is at least partly because of genetic factors. Animal models, especially rodents, have been developed to investigate the different symptoms of ASD. Social deficits can be investigated for example via the three-chamber social approach task or a play paradigm (Wöhr & Scattoni, 2013). In the three-chamber task the rodent can freely move around the apparatus and can chose to spend either more time with a strange stimulus rodent under a wire cup or with an object in the other compartment, whereas during a play task two unfamiliar rats can interact freely with each other in a neutral arena. The second core symptom of impairments in communication can be investigated by means of USV. Since ASD is a neurodevelopmental disorder, well known in ASD research are experiments with pup USV where the pups are typically placed outside the nest and the mother has to retrieve them. Unusual calling patterns or call parameters emitted by the pups (i.e. differing mean peak frequency) will prevent the mother from retrieving the pups back into the nest. This allows research of communicational deficits from a very early age on. Communication between males and females can be studied via scent-marking experiments, where solely female urine induces a USV response in the male recipient (Wöhr et al., 2011). Playback studies serve also as a great tool to investigate communicational deficits in rodents, as many studies demonstrated, e.g. that female-induced USV attract females (Hammerschmidt et al., 2009). The third symptom of ASD is repetitive and stereotyped behavior and can be studied in tests like marble burying or self-grooming, where the behavior of a rodent is scored in a neutral arena. Other core features of ASD are the insistence of sameness and restricted interest that can be seen in humans. In rodent models this can be investigated via reversal learning tasks like the Morris Water Maze or on a T-maze, whereas restricted interest can be shown via the nose hole poking task or the novel object task (Wöhr & Scattoni, 2013).

In a first genetic rat model for ASD, reduced social behavior could be detected (Berg et al., 2018). So far, only mouse models for ASD existed, but due to the more pronounced social behavior and social communication, the rat represents a better model to study neurodevelopmental disorders like ASD. During the 50-kHz USV playback paradigm in a rat model for ASD, namely the *Shank3*-deficient rat, *Shank3* null mutants showed reduced behavioral response (Berg et al., 2018). All genotypes of both sexes exhibited a strong social approach towards the 50-kHz USV playback. However, only wildtype and heterozygous animals kept exploring the proximal arms of the radial maze afterwards, whereas the null mutants did not. Regarding response calls towards 50-kHz USV playback, no genotype difference could be detected, but males emitted higher rates of response calls. All rats emitted 50-kHz as well as 22-kHz USV. The authors interpreted the 22-kHz USV response calls as an expression of frustration, which is in line with our frustration hypothesis about response calls. They drew the conclusion that the decreased behavioral response towards 50-kHz USV playback but no difference in response call emission might be due to "deficits in receptive but not expressive communication in this *Shank3* rat model, probably linked to reduced social motivation".

Besides the described paradigms to separately test the core symptoms of ASD in rat and mouse models, the repeated 50-kHz USV playback paradigm including the measurement of response calls could combine all these different tests. The social approach towards the 50-kHz USV represents intact social behavior, the emission of response calls depicts the ability of proper communication, and the repeated playback tests memory and learning.

In addition to the models for ASD, a newly developed rat model for AS reported various results during 50-kHz USV playback as well as response calls towards it. AS is a neurodevelopmental disorder caused through a deletion of the maternal *UBE3A* gene (Kishino et al., 1997; Matsuura et al., 1997; Chamberlain et al., 2010). Characteristic symptoms include developmental delay, impaired communication, ataxia, seizures, deficits in motor and balance, poor attention, microcephaly, and intellectual disabilities (Williams, 2005; Williams et al., 2010; Buiting et al., 2016). A newly developed rat model with a deletion of the maternal *UBE3A* gene (heterozygous *Ube3a* mat-/pat+ animals) leads to excessive laughter-like vocalizations as well as impaired communication, motor deficits, and microcephaly and seizures in rats (Berg et al., 2020, 2021a). Furthermore, playback studies in the AS rat model showed significantly decreased social approach behavior during 50-kHz USV playback in heterozygous *Ube3a* rats (Berg et al., 2020). Additionally, pro-social behavior can be tested not only during an initial playback but also with the repeated playback paradigm. The advantage of the repeated playback is that not only social approach behavior but as well learning and memory mechanisms can be studied (Wöhr & Schwarting, 2012). This is why the repeated playback paradigm was also applied in a rat model for AS. Preliminary results in the repeated playback paradigm in *Ube3a* maternal deletion rats showed a sex effect (unpublished data). Regardless genotype, female rats did not habituate whereas male rats habituated. Since in earlier studies the insulin-like growth factor II (IGF-II) proved to enhance cognitive and social learning in ASD-mouse models (Stern et al., 2014; Steinmetz et al., 2018), I tested whether IGF-II would be effective against learning deficits in the *Ube3a* deletion rat model for AS. I hypothesized that the administration of IGF-II before the first test would enhance memory and learning of the 50-kHz USV playback in the otherwise learning deficient heterozygous *Ube3a* rats in both sexes; hence in the first test the rats would approach the 50-kHz USV playback and in the retest, they would memorize the playback and habituate. The results showed that a genotype and a sex effect seemed to be present in female heterozygous *Ube3a* animals exhibiting habituation in the retest after IGF-II administration prior the first test. Female *Ube3a* wildtype rats still showed no habituation. Among male rats, wildtype animals showed an enhanced habituation during the retest, whereas male heterozygous *Ube3a* rats did not habituate, representing the learning deficits of the phenotype and IGF-II not being able to rescue this impairment. Furthermore, treatment with IGF-II was not able to rescue social communication deficits in heterozygous *Ube3a* rats during playback of 50-kHz USV playback

(Berg et al., 2021b). Additionally, the treatment with IFG-II was also not able to improve deficits in motor learning in rats, neither in mice (Berg et al., 2021b). The authors concluded that systemic treatment with IGF-II in rodent models of AS is difficult to generalize to humans and that it "does not profoundly improve behavioral deficits" (Berg et al., 2021b).

In addition to the results of social approach towards 50-kHz USV playback in the AS rat model, response calls towards 50-kHz playback were also detected (Berg et al., 2021a). During 50-kHz USV playback wildtype as well as heterozygous *Ube3a* rats emitted 50-kHz USV and short 22-kHz USV. There was no significant difference between the genotypes regarding duration or mean peak frequency, indicating that response calls towards 50-kHz UV playback are a reliable phenomenon and are in line with our results about response calls (Berg et al., 2021a). Nevertheless, a more detailed analysis of response calls in heterozygous *Ube3a* rats would be beneficial.

Besides the neurodevelopmental disorders ASD and AS, patients with schizophrenia also show impairments of communication represented by verbal learning memory (Nuechterlein et al., 2004). The Measurement and Treatment Research to Improve Cognition in Schizophrenia (MATRICS) initiative was founded due to the lack of effective treatments against Schizophrenia by bringing together academic, industrial, and governmental institutions to advance this issue (Marder et al., 2004; Young et al., 2009). This initiative focused on the seven cognitive domains being deficient in patients with schizophrenia and how to best model them in preclinical test batteries. Most of the domains (attention/vigilance; working memory; reasoning and problem solving; processing speed; visual learning and memory; verbal learning and memory; and social cognition) can be modeled by already existing animal models. For example, the novel object recognition test is a tool to investigate visual learning and memory in preclinical studies of schizophrenia (Green et al., 2004; Nuechterlein et al., 2004). Since language and speech are not found in any other animal besides *Homo sapiens* (Fitch, 2018), this makes it very difficult to address it in preclinical studies. Even though rodents have a wide variety of communication, homologous structures to language are arguable. MATRICS came to the conclusion that probably olfactory communication might be the best modality to represent the domain of verbal learning and memory, since processing specific odors through the olfactory cortex could be similar to verbal processing in the auditory cortex (Young et al., 2009). However, this is not an exact representation of the domain they aimed to assess. Therefore, the repeated playback paradigm would be well suited to model the domain of verbal learning and memory. Besides the social and acoustic aspects of the 50-kHz USV paradigm, the repeated playback also incorporates learning and memory mechanisms, what had been shown by pharmacologically inducing amnesia after the first playback test. This led to a repeated approach during the retest and inhibited habituation (Wöhr & Schwarting, 2012). USV are often used in research to investigate communication deficits and speech impairments in neurodevelopmental as well as neuropsychiatric disorders (Fujita et al., 2008; Scattoni et al., 2009; Wöhr et al., 2011). Comparing USV communication with speech has to be approached with caution, even though it is broadly thought that USV represent affective states and that 50-kHz USV might be a precursor of human laughter (Panksepp & Burgdorf, 2003), there is no definite evidence that USV might be similar to speech. Nevertheless, the repeated playback paradigm, especially when also considering response calls representing reciprocal communication, seems to be a good tool to investigate speech and deficits in verbal learning and memory in preclinical studies of schizophrenia.

Another rat model for neuropsychiatric disorders is the *Cacna1c* model. *CACNA1C* is a cross-disorder risk gene associated with several neuropsychiatric disorders such as affective disorders as major depressive disorder, bipolar disorder and neurodevelopmental disorders like ASD, and schizophrenia (Wöhr et al., 2021). Several deficits have been detected in heterozygous *Cacna1c* (+/-) rats compared to wildtype rats (+/+). These deficits include impairments in pro-social 50-kHz USV communication in male (Kisko et al., 2018) as well as female rats (Redecker et al., 2019). Additionally, sex-dependent (Braun et al., 2018) and long-term (Braun et al., 2019) effects of *Cacna1c* haploinsuffiency have been detected in object recognition, spatial memory and reversal learning tasks. Besides the behavioral deficits due to *Cacna1c* haploinsuffiency, lacks in adult neurogenesis in the prefrontal cortex and the hippocampus have been detected (Redecker et al., 2020) as well as mitochondrial resilience against oxidative stress have been detected (Michels et al., 2018, 2019). These various findings on a behavioral as well as a cellular and a molecular level show how effective rat models for neuropsychiatric disorders are in order to learn more about the disorders and find possible cures against them. The repeated 50-kHz USV playback paradigm including the measurement of response calls could be a good tool to further investigate impairments in learning and memory as well as social and communicational deficits in the *Cacna1c* rat model.

Taking these findings into consideration, the repeated playback paradigm represents a good tool to investigate not only fundamental mechanisms of social learning and memory, but also enables researchers to investigate models for neurodevelopmental or neuropsychiatric disorders, such as ASD, AS, schizophrenia, or affective disorders.

To further investigate the reasons for habituation or the function of response calls, several experimental approaches could be conducted. Regarding the already mentioned limitations, we do not know what the exact valence of the playback stimuli means to the rats. Therefore, it would be interesting to change the playback sequence in the retest compared to the first test. This might lead to an inhibition of habituation, because the animals are not familiar with the type of 50-kHz USV stimuli. To further investigate the function of response calls, playback of the response calls with lower frequencies (<32 kHz) could be carried out. The hypotheses for this experiment could be that approach behavior is induced, but that there might be an age difference and only juvenile but not adult rats exhibit social approach towards playback of lower frequency response calls.

Concluding remarks

The brain and how it influences behavior and communication is and remains one of the greatest mysteries to be solved completely. Every single step, still so small, brings human kind further to understand behavior and communication and the underlying neuronal mechanisms. Not only to understand the basic system, but also to find cures against neurodevelopmental and neuropsychiatric disorders. Overall, the results of this dissertation showed how to overcome the habituation phenomenon and characterized response calls towards 50-kHz USV playback and their feasible function. This opens doors for future research not only about social behavior, but also investigating learning and memory as well as reciprocal communication during one paradigm, the repeated playback paradigm. It also offers a unique opportunity to overcome the limitations of only being able to conduct the playback paradigm once due to habituation, as well as studying the rat as the receiver and the sender at the same time. The application of this paradigm further suggests a great tool to study neuropsychiatric or neurodevelopmental disorders where social and reciprocal communication as well as learning and memory are impaired.

Publications

Study I:

Limited generalizability, pharmacological modulation, and state-dependency of habituation towards pro-social 50-kHz calls in rats.

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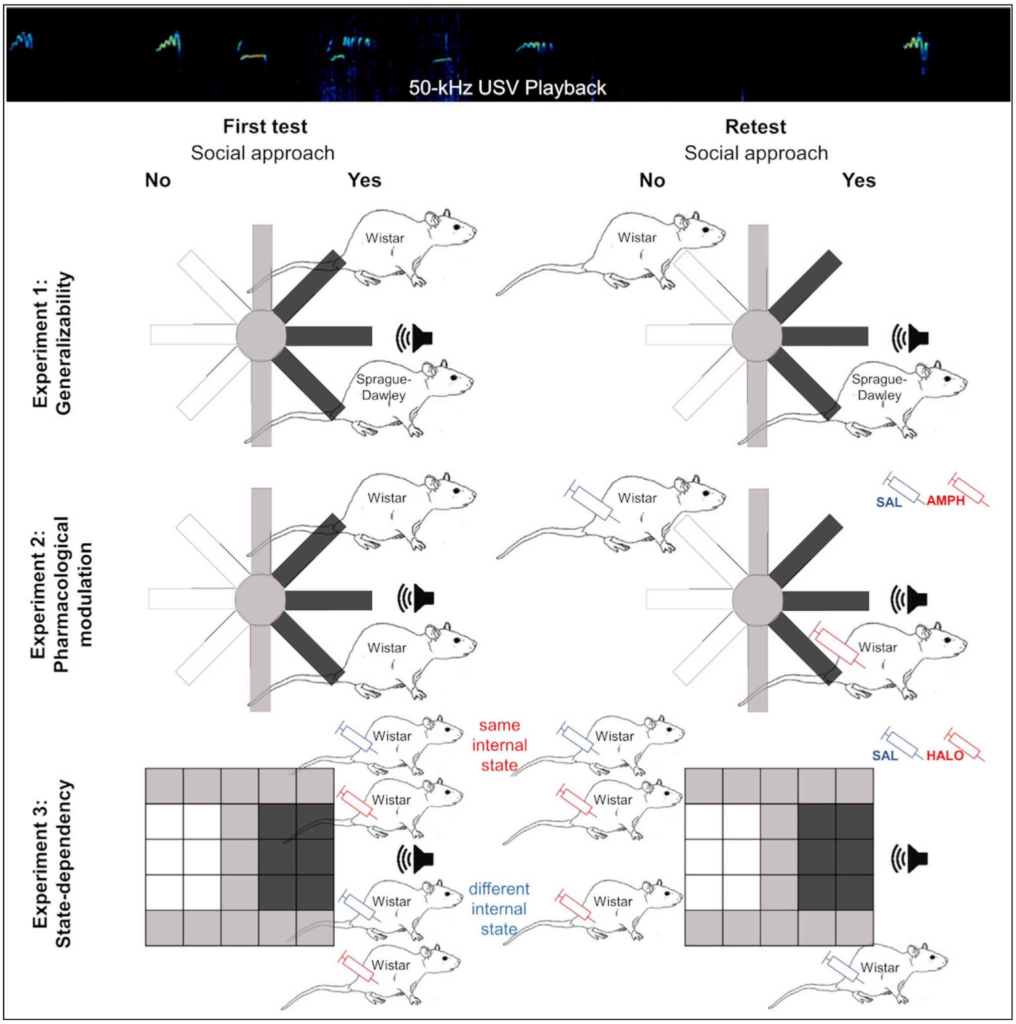

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Highlights

Rats display social approach in response to playback of pro-social 50-kHz calls

Repeated playback leads to habituation with limited generalizability

Habituation can be overcome by amphetamine treatment

Habituation depends on the subject's internal state

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Article

Limited generalizability, pharmacological modulation, and state-dependency of habituation towards pro-social 50-kHz calls in rats

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SUMMARY

Communication constitutes a fundamental component of mammalian social behavior. Rats are highly social animals and emit 50-kHz ultrasonic vocalizations (USV), which function as social contact calls. Playback of 50-kHz USV leads to strong and immediate social approach responses in receiver rats, but this response is weak or even absent during repeated 50-kHz USV playback. Given the important role of 50-kHz USV in initiating social contact and coordinating social interactions, the occurrence of habituation is highly unexpected. It is not clear why a social signal characterized by significant incentive salience loses its power to change the behavior of the receiver so rapidly. Here, we show that the habituation phenomenon displayed by rats in response to repeated playback of 50-kHz USV (1) is characterized by limited generalizability because it is present in Wistar but not Sprague-Dawley rats, (2) can be overcome by amphetamine treatment, and (3) depends on the subject's internal state.

INTRODUCTION

Throughout the animal kingdom, social behavior consists of a diverse set of often dynamic interactions between animals, ranging from basic attraction processes involved in the formation of simple aggregations to life in complex societies characterized by cooperation and competition. A central element to all major forms of social behavior is the transfer of information between sender and receiver through social signals coordinating social interactions. Communication constitutes a fundamental component of mammalian social behavior (for reviews see Hauser, 1996; Bradbury and Vehrencamp, 2011).

Rats are highly social animals and use several routes of communication, including sound. Most of their vocalizations are in the ultrasonic range, termed ultrasonic vocalizations (USV), which are known to serve as situation-dependent socio-affective signals (for reviews see Brudzynski, 2013; Wöhr and Schwarting, 2013). As juveniles and adults, rats emit two major call types. In aversive situations, such as predator exposure, 22-kHz USV occur, which probably express distress and serve as alarm signals to others (e.g. Blanchard et al., 1991; Fendt et al., 2018; Olszyński et al., 2020). In appetitive situations, for example rough-and-tumble play or mating, 50-kHz USV are emitted (e.g. Knutson et al., 1998; Burgdorf et al., 2008), which are thought to reflect the sender's positive affective state (''rat laughter''; Panksepp, 2005) and which initiate or maintain contact among conspecifics and coordinate social interactions (e.g. Siviy and Panksepp, 1987; Brudzynski and Pniak, 2002; Panksepp and Burgdorf, 2003; Schwarting et al., 2007; Wöhr et al., 2008; Łopuch and Popik, 2011; Kisko et al., 2015a and 2015b).

The signal features of such calls can efficiently be studied by using playback techniques, and for that purpose we had established a playback paradigm to examine the behavioral effects of presenting 22-kHz or 50-kHz USV to rats (Wöhr and Schwarting, 2007). Several studies have shown the effectiveness of this paradigm in investigating approach behavior in response to playback of 50-kHz USV in male and female Wistar rats, supporting the notion that they function as social contact calls (e.g. Wöhr and Schwarting, 2009; 2012; Willadsen et al., 2014; Seffer et al., 2015; Brenes et al., 2016; for reviews see Wöhr et al., 2016; Schwarting et al., 2018). Importantly, we found that approach occurs specifically in response to signals with frequencies typical for 50-kHz USV, because no such responses were observed when rats were exposed to background noise or 22-kHz USV (e.g. Wöhr and Schwarting, 2007; 2012; Fendt et al., 2018; Wöhr et al., 2020). Also, 50-kHz USV effectiveness is dependent on the animals' developmental stage, because approach proved

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to be clearly more pronounced in juvenile than adult rats (Wöhr and Schwarting, 2007), which is in line with the important pro-social role of 50-kHz USV during juvenile rough-and-tumble play (Knutson et al., 1998).

Neurochemical studies were performed and showed that social approach induced by 50-kHz USV is related to neuronal activation and increased dopamine (DA) release in the nucleus accumbens (Sadananda et al., 2008; Willuhn et al., 2014), a brain area well known for its critical role in motivated behavior, especially as an ''interface between motivation and action'' (Mogenson et al., 1980). Pharmacological studies using treatment with systemic d-amphetamine (AMPH) supported this relationship, since we found that the catecholaminergic agonist AMPH dose-dependently enhanced approach to playback of 50-kHz USV (Engelhardt et al., 2017, 2018). Also, we obtained evidence for an involvement of opiate mechanisms (Wöhr and Schwarting, 2009), with the agonist morphine promoting and the antagonist naloxone reducing approach. Finally, we and others successfully used 50-kHz USV playback to gauge the effects of environmental manipulations (Seffer et al., 2015; Brenes et al., 2016), and applied this paradigm to detect social communication deficits in various disease models, including genetic rat models with relevance to autism spectrum disorder (Kisko et al., 2018, 2020; 2020; Pultorak et al., 2016; Berg et al., 2018, 2020).

Importantly, most of the abovementioned evidence is based on results obtained during a first exposure to 50-kHz USV playback, since the prominent social approach response induced during the first exposure substantially declines with repeated 50-kHz USV playback even when performed several days later (Wöhr and Schwarting, 2012; Schönfeldet al., 2020). Given the important role of 50-kHz USV in initiating social contact and coordinating social interactions ranging from rough-and-tumble play to mating, the occurrence of habituation is highly unexpected and it is not clear why a social signal characterized by significant incentive salience loses its power to change the behavior of the receiver so rapidly. In fact, the reasons for this habituation phenomenon are largely unknown, but the effect appears to be memory-dependent since habituation in the retest was prevented by an amnesic drug, namely the muscarinic antagonist scopolamine, administered after trial to the first 50-kHz USV playback (Wöhr and Schwarting, 2012).

In light of this rather sparse evidence regarding habituation in response to repeated 50-kHz USV playback, we started a series of three experiments with the aim to gain a better understanding of the habituation phenomenon by assessing generalizability, pharmacological modulation, and state-dependency (Figure 1). Of note, in all experiments, we additionally used our standard acoustic control procedure, namely presentation of a series of noise stimuli with durations and amplitude modulations matching those of the original 50-kHz USV, which should lead to either no approach or even slight avoidance of the sound source.

Experiment 1: Because initial playback studies were largely based on Wistar rats as subjects, it is not known whether this phenomenon generalizes to other outbred stocks (commonly referred to as strains; Claassen, 1994), especially the much-used Sprague-Dawley rats. Although we also rely on Sprague-Dawley rats in some of our genetic disease models (Kisko et al., 2018, 2020), it is unclear whether they respond in similar ways as Wistar rats to repeated 50-kHz USV playback, i.e., whether they also habituate during the retest. To assess generalizability, we compared the behavioral response patterns of male Sprague-Dawley with Wistar rats when exposed to 50-kHz USV playback during a first test and a retest several days later.

Experiment 2: While it is known that systemic treatment with AMPH enhances approach to 50-kHz USV playback when given prior to the first test (Engelhardt et al., 2017, 2018), it is unclear whether it would act in a similar way in a retest several days later when rats show habituation to playback of 50-kHz calls, i.e., whether it would override the habituation phenomenon. Pharmacological modulation was analyzed by comparing Wistar rats treated with either AMPH (2.5 mg/kg) or saline (SAL) before undergoing a retest with playback of 50-kHz USV.

Experiment 3: Given that the habituation phenomenon appears to rely on intact memory (Wöhr and Schwarting, 2012) and memory retrieval was shown to be state-dependent (Radulovic et al., 2017), the habituation phenomenon might be state-dependent. By specifically manipulating the subject's internal state during the first test and/or retest, we either induced a match or a mismatch of the subject's internal state during acquisition, i.e., the first test and retrieval, i.e., the retest. A match is believed to facilitate memory retrieval and intact memory retrieval is expected to lead to the habituation phenomenon. In contrast, a mismatch is believed to hinder memory retrieval and impaired memory retrieval is expected to lead to a lack of the habituation phenomenon. In other words, it is expected that a mismatch of the subject's internal state results in limited access to the memory of the first test during the retest and that rats with limited

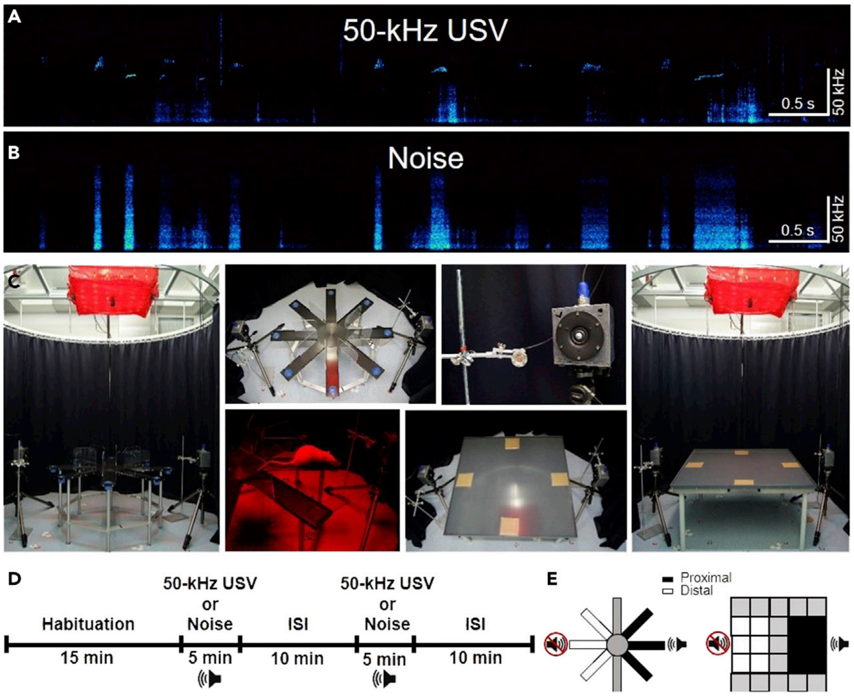

Figure 1. Acoustic stimuli and experimental setups

(A) Spectrogram of the sequence of 50-kHz USV used for playback. 50-kHz USV had been recorded from an adult male Wistar rat during exploration of a cage containing scents from a recently separated cage mate.

(B) Spectrogram of the sequence of noise used for playback. Time- and amplitude-matched noise was generated with SASLab Pro (Avisoft Bioacoustics) through replacing each given 50-kHz USV in the original natural 50-kHz USV stimulus by noise with durations and amplitude modulations matching to those of the original 50-kHz USV. Both stimuli were presented in a loop for 5 min each.

(C) Setup of the 50-kHz USV radial maze playback paradigm (left) and the 50-kHz USV platform playback paradigm (right). The elevated eight arm radial maze and the elevated squared platform were each equipped with two symmetrically positioned ultrasonic microphones and loudspeakers opposite of each other. Behavioral responses were recorded with a video camera positioned above maze and platform. Experiments were conducted under dim red light.

(D) Timeline of the three playback experiments. After a habituation phase of 15 min, either 50-kHz USV or noise stimuli were presented in a counterbalanced manner for 5 min. The inter stimulus interval (ISI) of 10 min was followed by a second stimulus presentation different to the first one. Each experiment ended with an additional ISI of 10 min.

(E) Schematic representation of the radial maze and the platform with proximal arms/zones (black) close to the active speaker on the right side and distal arms/zones (white) opposite the active speaker. The inactive speaker served as a visual control.

access to the relevant memory trace behave similar to rats lacking such a memory trace due to treatment with an amnesic drug (Wöhr and Schwarting, 2012). To systematically manipulate the subject's internal state, the DA D2 receptor antagonist haloperidol (HALO) was used as a pharmacological tool. In previous studies with a different design and testing environment (Tonelli et al., 2018a, 2018b; Melo-Thomas et al., 2020), we induced catalepsy with this drug and found that 50-kHz USV playback was effective in temporarily releasing rats from their cataleptic state and to approach the sound source. Here, we applied a fully balanced design, i.e., we gave either HALO (0.5 mg/kg) or SAL prior to the first test and/or the retest several days later. We asked whether the DAergic antagonist impairs 50-kHz USV-dependent approach during either test and, by doing so, whether the habituation phenomenon is state-dependent (e.g., Girden and Culler, 1937; for review see Radulovic et al., 2017); for example, whether habituation occurs in a SAL state in the retest, if the first test had been in the state of DAergic blockade.

RESULTS

Experiment 1: generalizability

Locomotor activity prior to playback (Figures 2A–2C): In the first playback test, locomotor activity declined prior to the start of playback (min 1-15; main effect time: $F_{14,476} = 7.652$, $p < .001$). This decline was

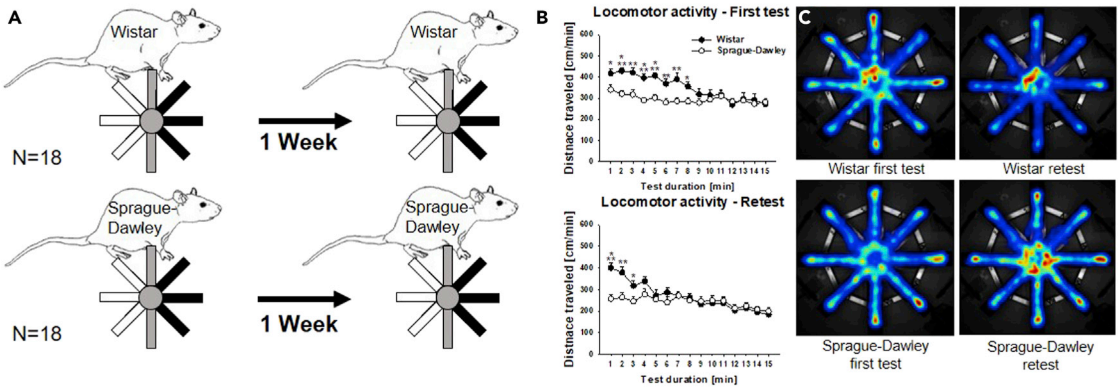

Figure 2. Generalizability – experimental design and locomotor activity

(A) Experimental design of the study: Comparison between Wistar and Sprague-Dawley rats in the repeated 50-kHz USV playback paradigm.

(B) Locomotor activity of Wistar (black circles) and Sprague-Dawley (white circles) rats during the habituation phase in the first 15 min of the first test (upper panel) and retest (lower panel).

(C) Average heat maps of locomotor activity during the initial 15 min of the first test and the retest for Wistar (upper panel) and Sprague-Dawley (lower panel) rats. Color coding reflects dwell time (red: most frequently visited locations, dark blue: least frequently visited locations). Please note that average heat maps reflect actual dwell times and not change scores.

Data are presented as means ± SEM (standard error of the mean). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, compared to Sprague-Dawley (locomotor activity).

particularly prominent in the initially more active Wistar rats, while Sprague-Dawley rats showed a rather moderate decline in locomotor activity (stock × time interaction: $F_{14,476} = 3.405$, $p < .001$), resulting in overall higher locomotor activity levels in Wistar than Sprague-Dawley rats (main effect stock: $F_{1,34} = 8.471$, $p = .006$). Likewise, prominent differences in the locomotor activity pattern were evident between stocks during the retest (time: $F_{14,476} = 13.192$, $p < .001$; stock: $F_{1,34} = 1.496$, $p = .230$; stock x time: $F_{14,476} = 4.916$, $p < .001$).

Responses to playback - First test (Figures 3A–3D and Figures 4A–4D): During the first test, playback of 50-kHz calls led to a strong social approach response in Wistar and Sprague-Dawley rats. This was reflected in increased proximal arm entries and time, together with decreased distal arm entries and time, compared to the preceding 5 min without stimulation (all p values <.05). None of these playback-induced change scores differed between Wistar and Sprague-Dawley rats (all p values >.05). In fact, a preference for proximal over distal arms was seen in Wistar and Sprague-Dawley rats, as evidenced by more proximal than distal arm entries and more time spent on proximal than distal arms (all p values <.05). Presentation of noise did not lead to social approach; rather, rats tended to decrease their arm entries and the time spent thereon, although typically not reaching statistical significance (all p values >.05; except distal arm entries in Wistar and Sprague-Dawley rats: $t_{17} = 3.128$, $p = .006$ and $t_{17} = 3.573$, $p = .002$; respectively). Again, there were no significant differences between Wistar and Sprague-Dawley rats (all p values >.05). Wistar and Sprague-Dawley rats did not display a preference for proximal arms during noise presentation (all p values >.05; except arm entries in Wistar rats: $F_{1,68} = 4.755$, $p = .044$).

Responses to playback - Retest (Figures 3A′–3D′ and Figures 4A′–4D′): During the retest, however, playback of 50-kHz calls did not lead to a social approach response in Wistar rats, consistent with the habituation phenomenon. Specifically, Wistar rats did not enter proximal arms more often during playback of 50-kHz calls than before playback ($t_{17} = 0.116$, $p = .909$). Moreover, they did not display an increase in the time spent on proximal arms ($t_{17} = 0.695$, $p = .496$). Likewise, distal arm entries were not affected by playback of 50-kHz calls ($t_{17} = 1.535$, $p = .143$), albeit the time spent on distal arms was lower during than before playback of 50-kHz calls ($t_{17} = 3.324$, $p = .004$). In fact, no preference for proximal over distal arms was seen in Wistar, as evidenced by similar levels of proximal and distal arm entries, together with similar levels of time spent on proximal and distal arms ($F_{1,68} = 0.053$, $p = .821$ and $F_{1,68} = 2.812$, $p = .112$; respectively).

In contrast, Sprague-Dawley rats still displayed a strong social approach response during the retest, suggesting limited generalizability of the habituation phenomenon. This was reflected by more proximal arm entries ($t_{17} = 3.803$, $p = .001$) and an increase in the time spent on proximal arms ($t_{17} = 4.336$, $p < .001$) during than before playback of 50-kHz calls, together with a decrease in the time spent on distal arms ($t_{17} = 3.387$,

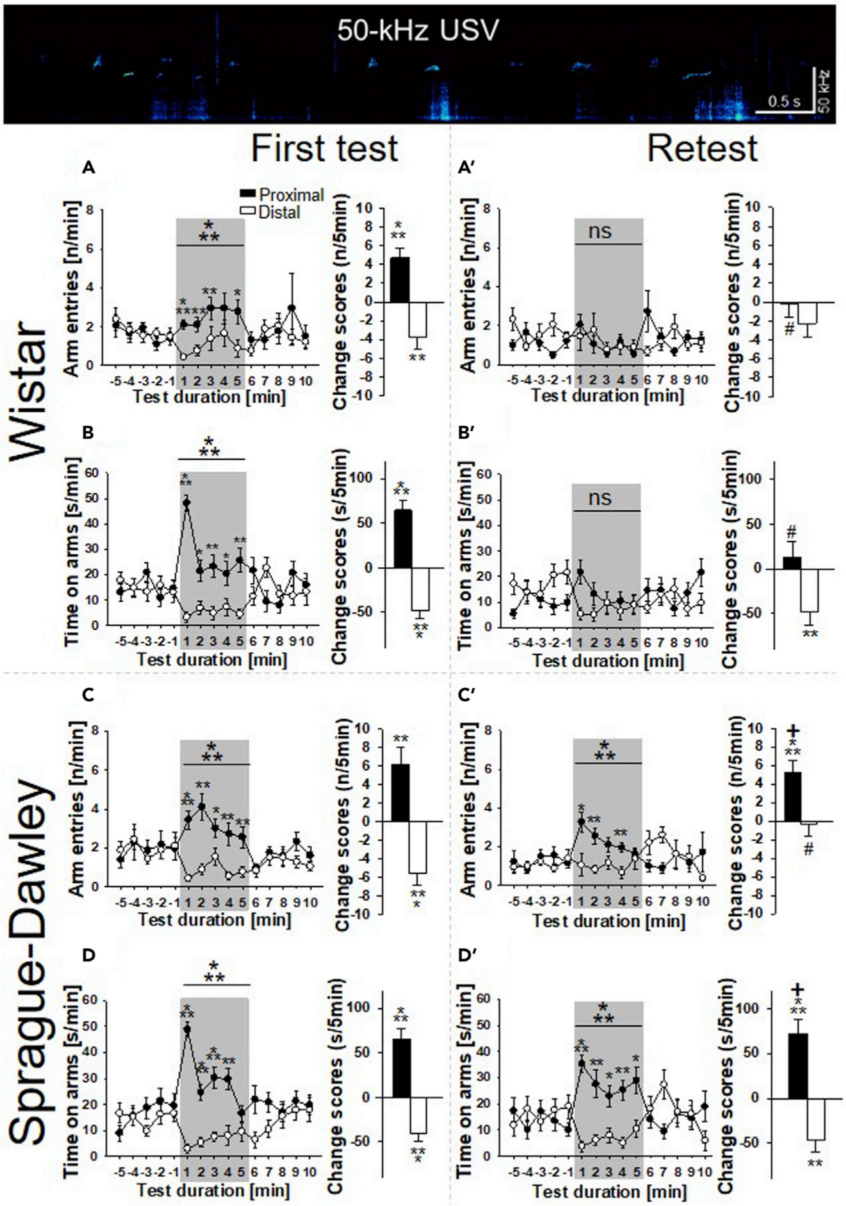

Figure 3. Generalizability – playback of 50-kHz USV

(A–D) Behavioral responses elicited by playback of 50-kHz USV during the first test. Behavioral responses were quantified as arm entries and time spent on arms in Wistar (A and B, respectively) and Sprague-Dawley rats (C and D, respectively). Wistar and Sprague-Dawley rats displayed clear social approach in response to playback of 50-kHz USV during the first test.

(A′–D′) Behavioral responses elicited by playback of 50-kHz USV during the retest. Behavioral responses were again quantified as arm entries and time spent on arms in Wistar (A′ and B′, respectively) and Sprague-Dawley rats (C′ and D′, respectively). Sprague-Dawley but not Wistar rats displayed clear social approach in response to playback of 50-kHz USV during the retest.

Left graphs depict time courses. Arm entries or time spent on arms proximal to (black circles) or distal from the sound source (white circles) during each min before, during (gray box), and after 50-kHz USV playback. Right graphs depict change scores. Change scores for arm entries or time spent on arms proximal to (black bars) or distal from the sound source (white bars) were calculated by subtracting entry and time measures during the 5 min before stimulus presentation from those during the 5 min of stimulus presentation. Data are presented as means ± SEM (standard error of the mean). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, compared to distal (time course) and to baseline (change scores). $^{\#}p < 0.05$, compared to first test (change scores). $^{+}p < 0.05$, compared to Wistar rats (change scores).

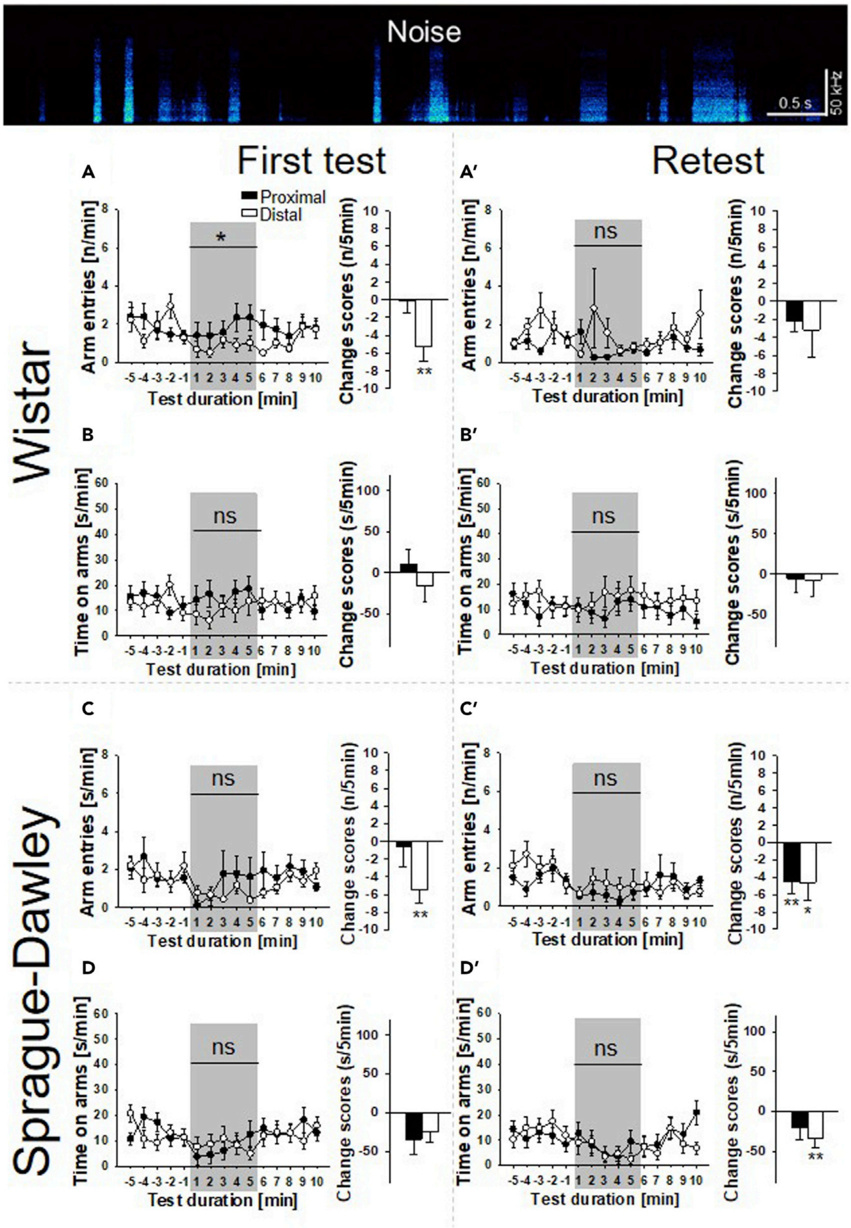

Figure 4. Generalizability - playback of time- and amplitude-matched noise

(A–D) Behavioral responses elicited by playback of time- and amplitude-matched noise during the first test. Behavioral responses were quantified as arm entries and time spent on arms in Wistar (A and B, respectively) and Sprague-Dawley rats (C and D, respectively). Noise did not lead to social approach during the first test.

(A′–D′) Behavioral responses elicited by playback of time- and amplitude-matched noise during the retest. Behavioral responses were again quantified as arm entries and time spent on arms in Wistar (A′ and B′, respectively) and Sprague-Dawley rats (C′ and D′, respectively). Noise did not lead to social approach during the retest.

Left graphs depict time courses. Arm entries or time spent on arms proximal to (black circles) or distal from the sound source (white circles) during each min before, during (gray box), and after noise playback. Right graphs depict change scores. Change scores for arm entries or time spent on arms proximal to (black bars) or distal from the sound source (white bars) were calculated by subtracting entry and time measures during the 5 min before stimulus presentation from those during the 5 min of stimulus presentation. Data are presented as means ± SEM (standard error of the mean). $^*p < 0.05$, compared to distal (time course) and to baseline (change scores).

$p = .004$), although this was not paralleled by a decrease in distal arm entries ($t_{17} = 0.262$, $p = .796$). In fact, a preference for proximal over distal arms was seen in Sprague-Dawley rats, as evidenced by more proximal than distal arm entries and more time spent on proximal than distal arms ($F_{1,68} = 18.917$, $p < .001$ and $F_{1,68} = 28.455$, $p < .001$; respectively).

Compared to the first test, changes in proximal arm entries and the time spent on proximal arms were less pronounced during the second playback in Wistar rats ($t_{17} = 2.389$, $p = .029$ and $t_{17} = 3.484$, $p = .003$; respectively), in line with the habituation phenomenon. Importantly, no such effects were observed in Sprague-Dawley rats and proximal arm entries and the time spent on proximal arm entries did not differ between the first and the second playback ($t_{17} = 0.413$, $p = .685$ and $t_{17} = 0.313$, $p = .758$; respectively) indicating that the social approach response during the second playback was as prominent as during the first playback. In fact, proximal arm entries and the time spent on proximal arms were higher in Sprague-Dawley than in Wistar rats during the second playback ($t_{17} = 2.706$, $p = .011$ and $t_{17} = 2.400$, $p = .022$; respectively), while distal arm entries and the time spent on distal arms did not differ ($t_{17} = 0.995$, $p = .327$ and $t_{17} = 0.097$, $p = .923$; respectively). Together, this indicates that Wistar rats do not show a social approach response during the retest, whereas in Sprague-Dawley rats a social approach response was still evident.

Of note, noise presentation did not lead to social approach and did not affect proximal and distal arm entries in Wistar rats ($t_{17} = 1.782$, $p = .093$ and $t_{17} = 1.015$, $p = .324$; respectively) but led to decreased proximal and distal arm entries in Sprague-Dawley rats ($t_{17} = 3.507$, $p = .003$ and $t_{17} = 2.223$, $p = .040$; respectively). Time spent on proximal and distal arms was not affected by noise playback (all p values $>.05$; except for time spent on distal arms in Sprague-Dawley rats: $t_{17} = 2.993$, $p = .008$). Wistar and Sprague-Dawley rats did not display a preference for proximal arms during noise presentation (all p values $>.05$).

Experiment 2: pharmacological modulation

First test - Without drug treatment (Figures 5D–5H): During the first test, playback of 50-kHz calls led to the expected pattern in Wistar rats, i.e., social approach, as reflected in increases in proximal arm behavior (arm entries: $t_{19} = 1.905$, $p = .036$; times spent: $t_{19} = 10.360$, $p < .001$), together with decreases in distal arm behavior (arm entries: $t_{19} = 2.164$, $p = .022$; times spent: $t_{19} = 2.160$, $p = .022$). Playback of noise led to decreases in the number of proximal and distal arm entries and the time spent on the arms (all p values $<.05$; except for time spent on proximal arms: $t_{19} = 1.605$, $p = .062$). Of note, the later AMPH and control rats did not differ (all p values $>.05$) and are therefore presented in a pooled manner.

Retest - Locomotor activity and USV emission after either AMPH or vehicle treatment (Figures 5A–5C): First, we tested whether AMPH treatment led to psychomotor activation on the radial maze, with locomotor activity (i.e., distance traveled) displayed during the initial 15 min of the retest serving as the index. We found clearly higher activity levels in AMPH-treated rats compared with SAL-treated controls (main effect drug: $F_{1,18} = 29.871$, $p < .001$; main effect time: $F_{14,252} = 15.507$, $p < .001$; time × drug interaction: $F_{14,252} = 2.898$, $p < .001$). Similar patterns were observed during the subsequent phases, including playback, where AMPH-treated rats continued to show higher locomotor activity levels than controls (details not shown).

Second, we tested whether AMPH led to the emission of 50-kHz calls. During the initial 15 min of the retest, i.e., prior to playback of 50-kHz USV or noise, four out of the ten rats treated with AMPH emitted 50-kHz calls, individually ranging between 23 and 567 calls in 15 min. These calls had the following mean features (±SEM): call duration 40 ± 0.8 ms, peak frequency 62.986 ± 4.219 kHz, and frequency modulation 23.666 ± 1.844 kHz. No 50-kHz calls were detected during this period in the other six AMPH-treated rats or any of the vehicle controls. Descriptively, the levels of AMPH-induced psychomotor activation during these 15 min were higher in the four vocalizing rats than the other six AMPH rats, which did not vocalize (means ± SEM: 7065 ± 737 vs. 6110 ± 544 cm), but these subgroups did not differ significantly from each other ($t_8 = 1.066$, $p = .318$).

Retest - Effects of AMPH on responses to playback (Figures 5D'–5H'): Finally, we asked whether AMPH treatment affected the responses to 50-kHz USV playback in the retest and whether it would override the habituation phenomenon. In SAL-treated animals, the habituation phenomenon was evident. Specifically, there were no increases in proximal arm entries in response to the playback of 50-kHz USV during the retest. If at all, there were decreases in both proximal and distal arm entries, yet not reaching statistical significance (all p values $>.05$). Regarding the time spent on arms, SAL-treated animals tended to show an

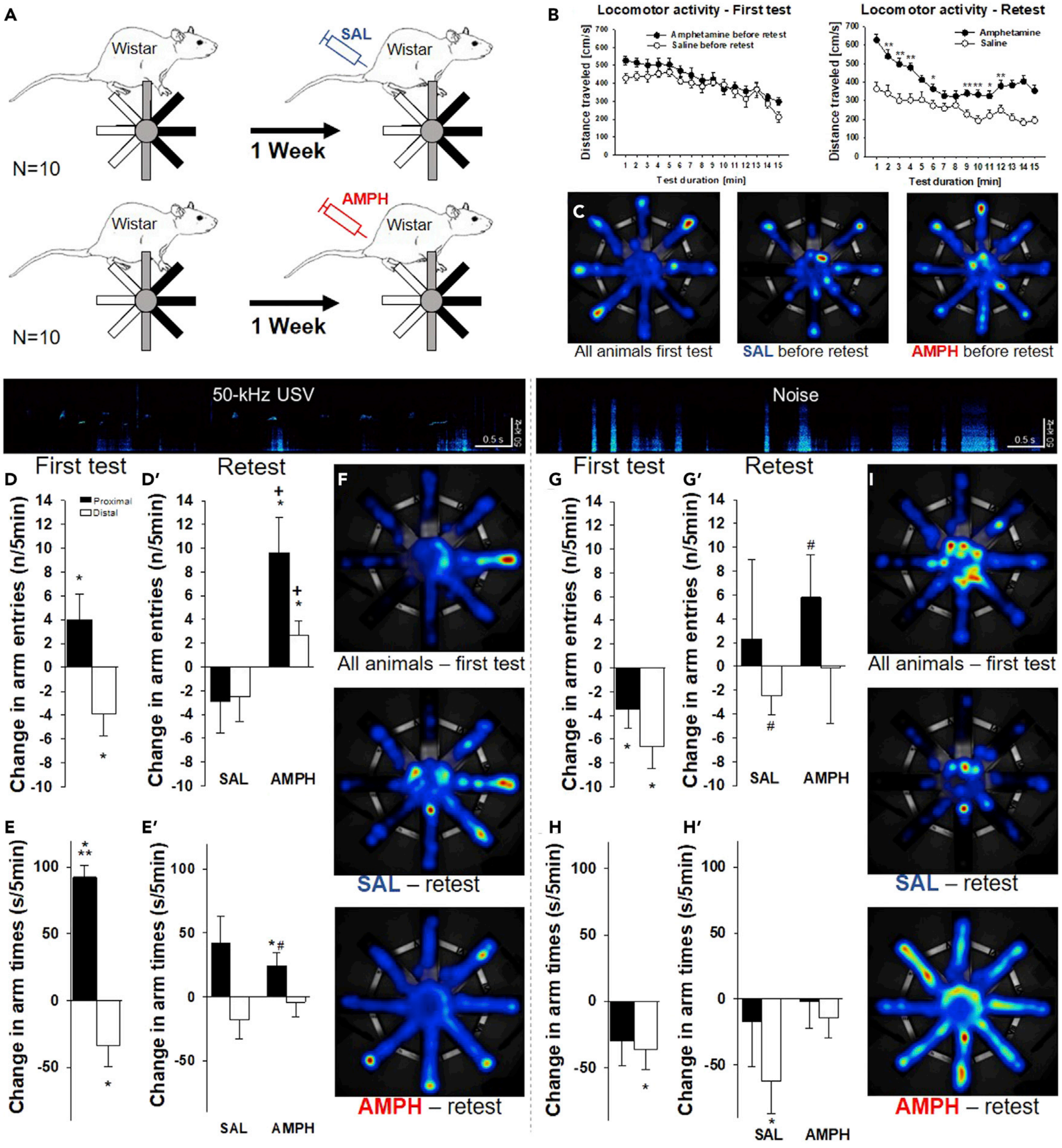

Figure 5. Pharmacological modulation

(A) Experimental design of the study: Pharmacological modulation in Wistar rats treated with d-amphetamine (AMPH) versus saline (SAL) before the retest in the repeated 50-kHz USV playback paradigm.

(B) Locomotor activity of rats treated with AMPH (black circles) or SAL (white circles) during the habituation phase in the first 15 min of the first test (left panel) and retest (right panel).

(C) Average heat maps of locomotor activity during the initial 15 min of the first test (left panel) and the retest for rats treated with AMPH (right panel) or SAL (center panel). Color coding reflects dwell time (red: most frequently visited locations, dark blue: least frequently visited locations).

(D and E) Behavioral responses elicited by playback of 50-kHz USV during the first test. Behavioral responses were quantified as arm entries and time spent on arms (D and E, respectively). Rats displayed clear social approach in response to playback of 50-kHz USV during the first test.

(D′ and E′) Behavioral responses elicited by playback of 50-kHz USV during the retest. Behavioral responses were again quantified as arm entries and time spent on arms (D′ and E′, respectively). AMPH-treated but not SAL-treated rats displayed clear social approach in response to playback of 50-kHz USV during the retest. Graphs depict change scores. Change scores for arm entries or time spent on arms proximal to (black bars) or distal from the sound source (white bars) were calculated by subtracting entry and time measures during the 5 min before stimulus presentation from those during the 5 min of stimulus presentation.

(F) Average heat maps during 50-kHz USV presentation. During the first test (upper picture), the red spot reflects strong social approach to the sound source located on the right side (3 o'clock). In the retest, SAL-treated rats exhibited less approach (center picture) and AMPH-treated rats (lower picture) seemed to have spent time exploring the whole maze, which is indicated by less red spots but equal distribution of activity over all arms.

Figure 5. *Continued*

(G–H') Behavioral responses elicited by playback of time- and amplitude-matched noise during the first test and retest. Noise did not lead to social approach.

(I) Average heat maps during noise presentation. During the first test (upper picture), rats spent most time in the center. During the retest, AMPH-treated rats (lower picture) showed more activity over the whole maze, whereas SAL-treated rats (center picture) exhibited far less locomotor activity. Please note that average heat maps reflect actual dwell times and not change scores.

Data are presented as means ± SEM (standard error of the mean). *p < 0.05, **p < 0.01, ***p < 0.001, compared to saline (locomotor activity) and to baseline (change scores). $^{\#}p < 0.05$, compared to first test (change scores). $^{+}p < 0.05$, compared to SAL-treated rats (change scores).

increase in the time spent on proximal arms in response to 50-kHz USV playback, while the time spent on distal arms was not affected (all p values >.05). These results are consistent with the habituation effects observed in Wistar rats in Experiment 1, albeit changes in proximal arm entries did not differ from the first test (t_9 = 0.737, p = .480), with a trend for the time spent on proximal arms (t_9 = 2.023, p = .074). Also, there were no significant changes to noise playback (all p values >.05; except for time spent on distal arms: t_9 = 2.551, p = .031).

In contrast to the SAL control group, however, AMPH-treated rats also displayed a social approach response during the retest, indicating that AMPH treatment can override the habituation phenomenon. Specifically, they showed increased arm entries during 50-kHz USV playback. These increases were observed in proximal (t_9 = 3.244, p = .010) and distal entries (t_9 = 2.299, p = .047), but tended to be more pronounced in case of proximal arm entries as compared to distal ones (t_9 = 2.145, p = .060). Moreover, rats treated with AMPH showed an increase in the time spent on proximal arms in response 50-kHz USV playback (t_9 = 2.370, p = .042), while the time spent on distal arms was not affected (t_9 = 0.378, p = .714). Compared to the first test, however, changes in the time spent on proximal arms was less pronounced during the second playback (t_9 = 3.971, p = .003), while proximal arm entries did not differ (t_9 = 0.488, p = .637). Consistent with the notion that AMPH treatment can override the habituation phenomenon, AMPH-treated rats displayed more proximal arm entries during 50-kHz USV playback than SAL-treated rats (t_{18} = 3.119, p = .006), albeit this was also the case for distal arm entries (t_{18} = 2.158, p = .045) but not the time spent on proximal and distal arms (t_{18} = 0.753, p = .461 and t_{18} = 0.733, p = .473; respectively). There were no significant changes in response to noise playback (all p values >.05).

Experiment 3: state-dependency

Effects of HALO on locomotor activity (Figures 6A and 6B): To test whether HALO led to the expected decrease in psychomotor activity, we analyzed locomotor activity during the initial 15 min of the first test and the retest in Wistar rats. In the first test, there was a decline in locomotor activity (main effect time: $F_{14,616}$ = 15.704, p < .001), which was more prominent in the SAL-treated groups (time × drug interaction: $F_{14,616}$ = 5.057, p < .001), most likely because HALO-treated groups displayed very low levels of locomotor activity from the beginning (main effect drug: $F_{3,44}$ = 49,543, p < .001). Post hoc tests did not yield differences within SAL- and HALO-treated groups (all p values >.05), respectively, but locomotor activity in HALO-treated groups was clearly lower than in SAL-treated groups (all p values <.001).

In the first 15 min of the retest, a largely similar picture was observed, i.e., a decrease over time (main effect time: $F_{14,616}$ = 16.979, p < .001), again primarily in the SAL groups, together with drug treatment effects (main effect drug: $F_{3,44}$ = 166.246, p < 0.001; time × drug interaction: $F_{42,616}$ = 4.062, p < 0.001). Post hoc tests again revealed that the HALO-treated rats displayed clearly lower locomotor activity than SAL-treated rats (all p values <.001). Now, however, there was also a difference between the two SAL groups, since the one which had received HALO rather than SAL in the first playback test showed more locomotor activity than the group which had received SAL in both tests (main effect drug: $F_{1,22}$ = 5.667, p = 0.026; time × drug interaction: $F_{14,308}$ = 1.600, p = 0.078). Likewise, the two HALO groups differed, with HALO-treated rats showing more locomotor activity if they had received SAL in the first playback test compared to those which also had received HALO (main effect drug: $F_{1,22}$ = 6.711, p = 0.017; time × drug interaction: $F_{14,308}$ = 8.284, p < 0.001).

Effects of HALO on responses to playback in the first test (Figures 6D–6H): In response to 50-kHz USV playback, SAL-treated animals (N = 24) showed a social approach response, as reflected by an increased number of entries into the zone next to the active speaker (t_{23} = 2.534, p = .019), together with a higher dwell time in the proximal zone (t_{23} = 6.288, p < .001) and a lower dwell time in the distal zone (t_{23} = 2.178, p = .040). In response to noise, proximal and distal zone entries were reduced (t_{23} = 2.524, p = .019 and t_{23} = 3.129,

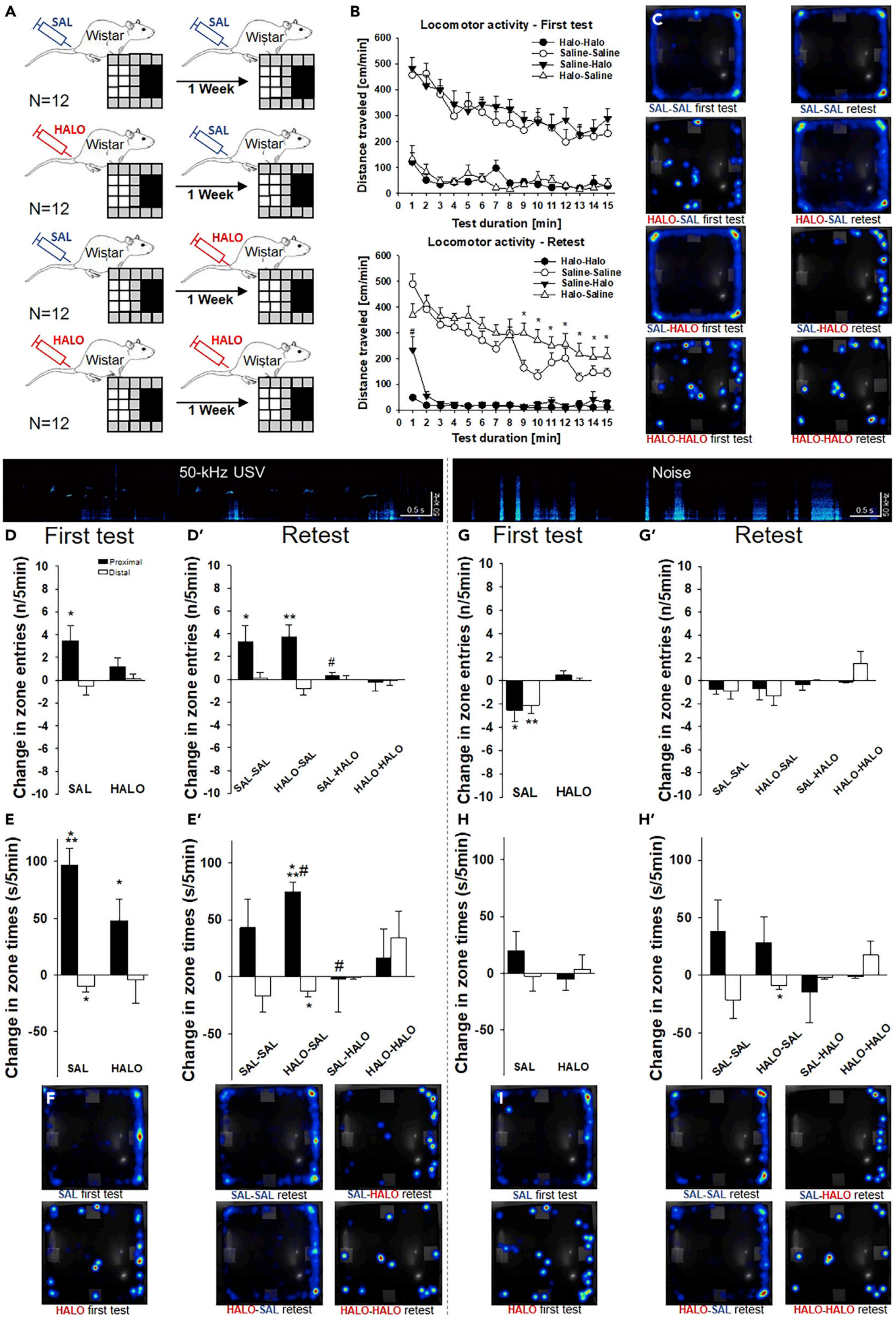

Figure 6. State-dependency

(A) Experimental design of the study: State-dependency in Wistar rats treated with haloperidol (HALO) versus saline (SAL) before the first test and/or before the retest in the 50-kHz USV playback paradigm. Wistar rats received either HALO or SAL before the first test on the platform. A week later, the same procedure was repeated with either the same injection (same internal state) or the other one (change of internal state).

Figure 6. ***Continued***

(B) Locomotor activity of rats treated with SAL-SAL (white circle), HALO-SAL (white triangle), SAL-HALO (black triangle), and HALO-HALO (black circle) during the habituation phase in the first 15 min of the first test (upper panel) and retest (lower panel).

(C) Average heat maps of locomotor activity during the initial 15 min of all treatment groups during the first test (left panel) and retest (right panel). Color coding reflects dwell time (red: most frequently visited locations, dark blue: least frequently visited locations). Of note, SAL-treated rats typically displayed activity along the edges of the platform with highest dwell times in the corners, while average heat maps of HALO-treated rats show distinct spots of activity, which is because individual rats sat mainly immobile in different spots of the platform.

(D and E) Behavioral responses elicited by playback of 50-kHz USV during the first test. Behavioral responses were quantified as zone entries and time spent in zones (D and E, respectively). SAL-treated but not HALO-treated rats displayed clear social approach in response to playback of 50-kHz USV during the first test.

(D′ and E′) Behavioral responses elicited by playback of 50-kHz USV during the retest. Behavioral responses were again quantified as zone entries and time spent in zones (D′ and E′, respectively). SAL-treated rats displayed clear social approach in response to playback of 50-kHz USV during the retest, if treated with HALO before, i.e., HALO-SAL, but not if treated with SAL before, i.e., SAL-SAL. HALO-treated rats, i.e., SAL-HALO and HALO-HALO, did also not display clear social approach in response to playback of 50-kHz USV during the retest. Graphs depict change scores. Change scores for zone entries or time spent in zones proximal to (black bars) or distal from the sound source (white bars) were calculated by subtracting entry and time measures during the 5 min before stimulus presentation from those during the 5 min of stimulus presentation.

(F) Average heat maps during 50-kHz USV presentation. During the first test (left panel), SAL-treated rats show strong social approach to the sound source located on the right side (3 o'clock). During the retest (right panel), rats treated with SAL in the retest spent time in the proximal zones as well. In general, average heat maps of HALO-treated rats show that individual rats rather sat in one position during stimulus presentation.

(G–H′) Behavioral responses elicited by playback of time- and amplitude-matched noise during the first test and retest. Noise did not lead to social approach.

(I) Average heat maps during noise presentation. SAL-treated rats displayed activity along the edges of the platform with highest dwell times in the corners similar to baseline, while average heat maps of HALO-treated rats show the usual 'spotted' activity. Please note that average heat maps reflect actual dwell times and not change scores.

Data are presented as means ± SEM (standard error of the mean). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, compared to saline (locomotor activity) and to baseline (change scores). $p < 0.05$, compared to first test (change scores).

p = .005, respectively), whereas changes in dwell times were not significantly affected. In HALO-treated rats (N = 24), there were no significant changes in zone entries and dwell times during 50-kHz USV playback, except for more time spent in the zone next to the speaker ($t_{23} = 2.538$, $p = .018$). Comparing SAL- and HALO-treated rats showed that changes in dwell time in the proximal zone tended to be lower in HALO-treated rats during 50-kHz USV playback ($t_{46} = 2.007$, $p = .051$). SAL-treated rats showed a trend for less proximal zone entries ($t_{46} = 1.917$, $p = .061$) and less distal zone entries ($t_{46} = 2.959$, $p = .005$) in response to noise.

Effects of HALO on responses to playback in the retest (Figures 6D′–6H′): In the retest, a clear social approach response to playback of 50-kHz USV was only seen in SAL-treated rats which had received HALO during the first test, indicating state-dependency of the habituation phenomenon. These rats displayed an increase in the number of entries into the proximal zone ($t_{11} = 3.563$, $p = .004$) and spent more time there in proximity to the active ultrasonic speaker during 50-kHz USV playback ($t_{11} = 9.006$, $p < .001$), together with less dwell time in the distal zone ($t_{11} = 2.523$, $p = .028$). Less dwell time in the distal zone was also evident during noise playback ($t_{11} = 2.322$, $p = .040$). No changes were detected in the other entry and time measures (all p values >.05). Interestingly, HALO-SAL rats spent even more time in the proximal zone during the second 50-kHz USV playback than during the first test ($t_{11} = 6.567$, $p < .001$; zone entries: $t_{11} = 1.534$, $p = .153$). In contrast, SAL-treated rats which had received SAL also before the first test did not display a clear social approach response. Although the number of proximal zone entries increased in response to 50-kHz USV playback ($t_{11} = 2.409$, $p = .035$), the time spent in proximity to the active ultrasonic speaker was not significantly higher than before playback ($t_{11} = 1.71$, $p = 0.115$), indicative of the habituation phenomenon. Proximal zone time did not differ between first test and retest in SAL-SAL rats ($t_{11} = 1.537$, $p = .152$; zone entries: $t_{11} = 0.213$, $p = .835$). None of the other entry or time measures yielded significant changes (all p values >.05). The two groups which had received HALO prior to the retest did not display social approach responses (all p values >.05). This was also reflected in prominent differences between experimental groups depending on whether they received haloperidol or not for proximal arm entries and the time spent on proximal arms ($F_{1,44} = 13.313$, $p < .001$ and $F_{1,68} = 4.827$, $p = .033$; respectively). However, SAL-SAL rats did not differ from HALO-SAL rats and SAL-HALO rats did not differ from HALO-HALO rats (all p values >.05). None of the other entry or time measures yielded significant changes (all p values >.05).

Response calls: During the first test, the majority of the rats emitted response calls during playback of 50-kHz USV (67%). A week later, during the retest, however, only a minority of the rats emitted response calls during playback of 50-kHz USV (17%; $chi^2 = 24.686$, $p < .001$). No rat emitted response calls during playback of noise during the first test or the retest (0%). The fact that the number of rats emitting response calls during playback of 50-kHz USV was clearly lower during the retest than the first test indicates that the habituation phenomenon does not only occur at the level of social approach behavior but also response calls. In fact, the habituation at the level of the response calls was so prominent that it occurred independently of whether drug treatment was changed between the first test and the retest in all groups (SAL-SAL: $chi^2 = 6.750$, $p = .009$; HALO-SAL: $chi^2 = 8.224$, $p = .004$; SAL-HALO: $chi^2 = 4.444$, $p = .035$; HALO-HALO: $chi^2 = 6.171$, $p = .013$).

DISCUSSION

Social contact calls play a prominent role in basic attraction processes between animals (Kondo and Watanabe, 2009). They function to initiate or maintain social proximity and group cohesion, e.g., by reuniting visually separated individuals. Fundamental for survival and reproduction, contact calls occur in a very large number of species independent of social structure. In line with their fundamental role, social contact calls are typically characterized by significant incentive salience since they lead to prompt and strong behavioral responses in the receiver. In a few cases, however, social contact calls were reported to rapidly lose the power to change the behavior of the receiver. For instance, in mice, Hammerschmidt et al. (2009) showed in a playback paradigm that male USV result in a prominent social approach response in females when presented the first time, but that this response vanished during a second presentation. Similarly, we found that playback of 50-kHz USV leads to strong and immediate social approach responses in receiver rats, but that this response is weak or even absent during repeated 50-kHz USV playback (Wöhr and Schwarting, 2012). Given the important role of 50-kHz USV in initiating social contact and coordinating social interactions, the occurrence of habituation is highly unexpected. It is not clear why a social signal characterized by significant incentive salience loses its power to change the behavior of the receiver so rapidly. The present series of three experiments aimed at gaining a better understanding of the habituation phenomenon by assessing generalizability, pharmacological modulation, and state dependency.

Experiment 1: generalizability

In the first playback test, juvenile male Wistar and Sprague-Dawley rats showed pronounced social approach to 50-kHz calls, as reflected in a higher number of proximal arm entries and more time spent on proximal arms at the cost of distal ones. No such effects were observed in case of noise, if at all, trends for decreases were observed, highlighting the specificity of the social approach response. These results are largely in line with several previous studies (Wistar: Wöhr and Schwarting, 2007; 2009; 2012; Seffer et al., 2015; Brenes et al., 2016; Engelhardt et al., 2017; 2018; Sprague-Dawley: Kisko et al., 2018; Berg et al., 2018; 2020) and show the robustness of the social approach response, which can also be observed in female Wistar and Sprague-Dawley rats (Willadsen et al., 2014; Berg et al., 2020; Kisko et al., 2018, 2020).

During the retest, performed one week after the first test, the result pattern changed substantially. As expected, Wistar rats did not show approach to 50-kHz USV playback. Both proximal arm entries and the time spent on proximal arms did substantially increase during the first test, but not the retest. In fact, proximal arm entries and the time spent on proximal arms during the retest were lower than during the first test. This behavioral change, which can be considered as habituation to repeated 50-kHz USV playback, is consistent with our previous findings (Wöhr and Schwarting, 2012). We interpreted this phenomenon as the result of a social acoustic memory initiated by the first playback experience, since the reduction of approach during the retest could be prevented by treatment with the amnesic drug scopolamine, administered immediately after trial to the 50-kHz USV playback. The psychological reasons for this effect are not clear yet. Probably, the rats learned that these social acoustic signals and approach to them were not followed by any social consequences, like encountering a conspecific. This information might then be retrieved during the retest and, as a consequence, leading to only weak or no approach during the second presentation.

In the first experiment, we asked whether the habituation phenomenon is dependent on stock since the majority of studies on the effects of repeated 50-kHz USV playback were conducted in Wistar rats. Interestingly, we did not obtain evidence for prominent habituation in Sprague-Dawley rats. In fact, in stark contrast to Wistar rats, Sprague-Dawley rats still displayed pronounced social approach during the retest. The increase in proximal arm entries and the time spent on proximal arms evoked by 50-kHz USV playback was clearly evident in Sprague-Dawley but not Wistar rats. Which factor(s) may have accounted for the result that Sprague-Dawley did not habituate to 50-kHz USV playback during the retest? One may be the kind of playback material: Both groups received a series of 50-kHz calls recorded from an adult male Wistar rat exploring an empty cage containing scents from a cage mate (Wöhr et al., 2008). Therefore, these calls may have had different informational value for Sprague-Dawley than Wistar rats. For instance, they may have been less familiar to Sprague-Dawley rats, which might decrease the likelihood of habituation. Several points, however, argue against this. For one, habituation in the retest with the same stimulus material as used here was recently found in Long-Evans rats (Schönfeld et al., 2020), indicating that the calls constituting the playback material do not have to be taken from the stock to be tested. Secondly, our Sprague-Dawley and Wistar rats, although housed in separate group cages, were kept at the same time in the same vivarium. Therefore, all rats should have had substantial experience with USV of their own stock and those of the other.

In a previous study (Schwarting, 2018), we compared 50-kHz USV emission between male Wistar and Sprague-Dawley rats in a cage test and during tickling. In the cage test, which might be more similar to the context of our present playback material, Sprague-Dawley and Wistar rats did not differ in call number, peak frequency, frequency modulation, or amplitude, which argues against the role of these acoustic features for our present stock-dependent outcomes. Moreover, we had shown before that replacing playback of natural 50-kHz USV by sine wave tones of identical durations, frequencies, and amplitudes also led to approach behavior of Wistar rats with similar strength as the present natural calls (Wöhr and Schwarting, 2007). These call features apparently do not account substantially to the calls' effectiveness in Wistar rats. Other 50-kHz USV features might still be critical, e.g., call durations which were found to be shorter in Sprague-Dawley than Wistar rats (Schwarting, 2018). Also, the distribution of calls over time may differ between stocks, but this factor has not yet been investigated to the best of our knowledge.

None of these acoustic factors, however, can explain why approach to 50-kHz USV playback was very similar between Wistar and Sprague-Dawley rats in the first test but differed in the retest. Therefore, other and subject-dependent factors need to be considered and here genotype might have accounted critically for the fact that Sprague-Dawley did not substantially habituate to 50-kHz USV playback during the retest. In the present context, stock differences in social behavior appear to be of particular relevance. Manduca et al. (2014a, 2014b) reported that Sprague-Dawley rats display substantially higher levels of rough-and-tumble play than Wistar rats. Interestingly, the heightened level of rough-and-tumble play in Sprague-Dawley rats was found to be paralleled by an increase in the emission of 50-kHz USV. Elevated levels of 50-kHz USV were not only seen during rough-and-tumble play but also during social and even cage exploration. It therefore appears possible that the lack of the habituation phenomenon in Sprague-Dawley rats is due to higher levels of social motivation translating into repeated attempts to establish contact with a conspecific emitting 50-kHz USV. However, it has to be noted that in a more recent study, an opposite pattern was obtained, with Wistar rats engaging more in rough-and-tumble play than Sprague-Dawley rats (Northcutt and Nwankwo, 2018), while only moderate differences between stocks were seen in another study (Himmler et al., 2014). Moreover, no stock differences in the emission of 50-kHz USV were seen when rough-and-tumble play was mimicked through tickling (Schwarting, 2018).

In this context, it is also interesting to note that the two stocks did not only differ in their general levels of locomotor activity on the maze, but also in terms of habituation to it, which was observable in Wistar but not Sprague-Dawley rats. Importantly, these effects occurred before presentation of the very first playback and were therefore not affected by it. Moreover, affective differences between Wistar and Sprague-Dawley rats might play a role, since Rex et al. (2004) and Rybnikova et al. (2018) considered Wistar to be more anxious (elevated plus-maze, holeboard), and Staples and McGregor (2006) judged Wistar rats to be more defensive in response to a predator odor than Sprague-Dawley rats. Therefore, one might expect less and not more locomotor activity in Wistar compared to Sprague-Dawley rats. Since a radial maze, as used here, has similarities with an elevated plus-maze, in that it is elevated and consists of a number of open arms, the test surely has some anxiogenic features, but lacks the safer enclosed arms of the plus-maze. Therefore, the animals cannot retreat and must cope otherwise with this environment. Interestingly, Walker et al. (2009) considered Wistar rats to display more novelty-seeking and more active coping than Sprague-Dawley rats, which might explain why the Wistar rats showed more locomotor activity on the radial maze, especially during the first minutes of exposure. While such stock-dependent dispositions might also explain why Wistar and Sprague-Dawley rats responded differently to repeated playback of 50-kHz calls, it is unlikely that the habituation phenomenon is driven by anxiety or stress. As anxiety and stress are expected to be highest during the exposure to a novel environment and thus during the first playback of 50-kHz USV, anxiety and stress would be supposed to inhibit social approach primarily during the first but not the second playback of 50-kHz USV. In other words, anxiety or stress would be expected to lead to a dishabituation but not a habituation phenomenon. In order to better understand the role of anxiety and stress, however, additional experiments are warranted and it would be interesting to test whether the habituation phenomenon occurs in less anxiogenic environments, such as a regular cage or an open field, and whether it depends on the light conditions, i.e., dim versus bright light.

Experiment 2: pharmacological modulation

In the second experiment, we investigated how systemic treatment with the psychostimulant AMPH affects the behavior of Wistar rats in the retest. For that purpose, we selected a drug dose which has repeatedly been shown to be effective in eliciting 50-kHz USV in juvenile and adult rats (Natusch and Schwarting, 2010; Pereira et al., 2014; Rippberger et al., 2015; Wöhr et al., 2015; Engelhardt et al., 2017, 2018), and to modulate approach in response to 50-kHz USV playback, at least when injected prior to the first test (Engelhardt et al., 2017, 2018). Treatments with AMPH prior to the retest have not been tested yet to our knowledge.

In the retest, AMPH led to an expected increase in psychomotor activity as measured during the 15 min prior to playback, since the AMPH group showed more locomotor activity than the vehicle group. Furthermore, 4 out of 10 AMPH-treated and none of the control rats emitted 50-kHz calls themselves during the 15 min prior to playback. The number of vocalizing rats was rather low compared to previous AMPH-studies but is probably due to the testing environment. Previous studies had shown that 50-kHz USV is more likely in subjectively safe situations (Natusch and Schwarting, 2010), whereas the open-armed radial maze might be too ambiguous (Engelhardt et al., 2017). Descriptively, the calling rats showed more locomotor activity than the other ones, but this pattern was not significant, which might be due to the small post hoc sample sizes of these subgroups. Alternatively, there might be no prominent relationship between AMPH-induced locomotor activity and 50-kHz calls (Ahrens et al., 2013; Taracha et al., 2014; Engelhardt et al., 2018), which, although closely linked to meso-limbic DA function, might at least partly be modulated by different neural mechanisms (Natusch and Schwarting, 2010).

While in the first test without any treatment, the rats showed the expected approach response to playback of 50-kHz USV, the evoked response in the retest was dependent on pharmacological treatment. In the retest, i.e., after either SAL or AMPH injection, SAL-treated controls did not display a clear approach response to 50-kHz USV playback, which is in line with the expected retest habituation response, as observed in Wistar rats in Experiment 1. In fact, lack of preference in SAL-treated controls during the retest was reflected in both measures, i.e., arm entries and times spent on arms. In case of AMPH, on the other hand, approach was still observed in terms of arm entries and times spent on arms, indicating that the drug prevented the otherwise typical habituation to repeated 50-kHz USV playback. However, while the increase in proximal arm entries and the time spent on proximal arms evoked by 50-kHz USV playback was clearly evident in AMPH-treated rats but not SAL-treated controls, the two experimental groups did not differ significantly from each other during the retest, limiting the conclusions that can be derived from these results. Moreover, the fact that AMPH also increased distal arm entries indicates that the observed results are at least partly driven by an unspecific stimulatory effect, which probably led to generally increased ambulation on the maze. Importantly, however, the increase in locomotor activity induced by AMPH did not conceal the approach effect to 50-kHz USV playback because locomotor activity was primarily directed toward the active ultrasonic speaker emitting 50-kHz USV, as reflected in a prominent increase in the time spent on proximal arms, while the time spent on distal arms remained unchanged. Although further evidence is needed, this is in line with our previous findings obtained during the first test and suggests that the present playback effects of AMPH "*did not simply reflect an unspecific byproduct of amphetamine-induced hyperactivity, but a specific enhancement in goal-directed social behavior*" (quoted from Engelhardt et al., 2017), especially the 'wanting' rather than the 'liking' component (Berridge et al., 2009) of social contact. These results again support the general hypothesis that the processing of 50-kHz USV in the receiver is strongly dependent on meso-limbic DA function in the brain (Willuhn et al., 2014), which is a critical substrate for "wanting" and approach (e.g., Mogenson et al., 1980; Berridge et al., 2009). In fact, meso-limbic DA signaling was previously shown to be involved in closing a perception-to-action-loop through linking mechanisms relevant for the detection of 50-kHz USV to behavioral responses, such as social approach. Specifically, Willuhn et al. (2014) showed that playback of 50-kHz USV can lead to phasic DA release in the nucleus accumbens similar to a food reward and that the evoked social approach response is positively correlated with accumbal DA release, but that the DA response vanishes with repeated 50-kHz USV playback together with the behavioral changes. This indicates that 50-kHz USV transiently exhibit a similar incentive salience and involve the same neural systems as a primary reward, but then dissipate this property rapidly – an effect that can be overcome by AMPH treatment, presumably by boosting meso-limbic DA signaling.

In contrast to the findings of Engelhardt et al. (2017), AMPH did not lead to enhanced avoidance during noise presentation. There, however, AMPH was tested during the first test, and not the retest as done here. Possibly, the noise effects found by Engelhardt et al. (2017) were due to an interaction between the drug and the novelty of the test situation. AMPH can also have anxiogenic effects (Pellow et al., 1985; Lapin, 1993; White et al., 1995), and these might be more prominent in an unfamiliar situation leading to avoidance of the arbitrary noise stimulus.

Experiment 3: state-dependency

In the third experiment, we tested whether the habituation phenomenon is state-dependent by systematically manipulating the subject's internal state through pharmacological treatment. By specifically manipulating the subject's internal state during the first test and/or retest, we were able to generate a mismatch of the subject's internal state during acquisition, i.e., the first test, and retrieval, i.e., the retest, which is known to hinder memory retrieval (e.g. Girden and Culler, 1937; for review see Radulovic et al., 2017). Because there is evidence indicating that intact memory function underlies the habituation phenomenon (Wöhr and Schwarting, 2012), impaired memory retrieval due to a mismatch of subject's internal states is expected to prevent the habituation phenomenon. In contrast to Experiment 2, which used a catecholaminergic agonist, an antagonist was used in Experiment 3, namely the D2 receptor antagonist HALO. Importantly, and in extension of the design used in Experiment 2, we varied the time points of injection, i.e., either before the first test and/or before the retest. Also, we used a different environment, namely a platform, since that had also been used in our previous playback studies where HALO-dependent outcomes were investigated (Tonelli et al., 2018a, 2018b; Melo-Thomas et al., 2020).

In general, HALO led to the expected decreases in locomotor activity (e.g., Campbell and Baldessarini, 1981; Wiley, 2008), as assessed by distances traveled during the initial 15 min of the first test and the retest. This effect was even stronger in the retest, which is in line with earlier findings, which were explained in terms of sensitization with repeated HALO treatment (Banasikowski and Beninger, 2012). Also, the outcome of HALO in the first test might have been affected by the novelty of the situation, a factor which can reduce DAergic drug effectiveness (Bardo et al., 1990).

Interestingly, drug treatment during the first test apparently affected SAL-behavior in the retest, since locomotor activity in the retest was lower in SAL-treated animals if they had also received SAL in the first test rather than HALO. Apparently, locomotor habituation in the retest was less pronounced in the HALO-SAL group, since the drug had prevented these animals from actively exploring the test environment in the first test, which made it necessary to explore more in the retest.

With respect to playback in the first test, SAL-treated rats showed responses to 50-kHz USV playback which, in qualitative terms, are largely similar to those of Experiments 1 and 2, namely increases in proximal zone entries and dwell times. This indicates that the effectiveness of 50-kHz USV playback to elicit approach cannot only be gauged by using a radial maze, but also by the more simplified platform version, where approach is evaluated by analysis of virtual zones in line with a recent report on social approach evoked by playback of 50-kHz USV in a home cage (Olszyński et al., 2020). Using the platform, we found that HALO partly blocked the approach to presentation of 50-kHz USV, which only led to an increase in proximal zone times but not proximal zone entries. The increase in zone times tended to be less pronounced than the one observed in SAL-treated rats. The inhibitory effect on entries was probably due to the pronounced inhibition of locomotor activity exerted by the DAergic receptor antagonist. In the previous studies (Tonelli et al., 2018a, 2018b) and the same dose of HALO, we found that 50-kHz USV playback released rats from drug-induced catalepsy and eventually even led to approach toward the acoustic signal source, i.e., an outcome which appears to be stronger than in the present case. These differential patterns are probably due to methodological reasons, since Tonelli et al. (2018a, 2018b) used a bar (placed centrally on the platform), on which the rats had to remain before playback, whereas in the present study they were tested without such a bar nor temporal restriction to the center. Despite these partial differences, one can conclude that approach to 50-kHz calls does not require functioning DA D2 receptors, but that its expression is moderated by them.

In the retest, a clear social approach response to playback of 50-kHz USV was only seen in SAL-treated rats, which had received HALO during the first test, indicating state-dependency of the habituation phenomenon. These rats displayed an increase in the number of entries into the proximal zone and spent more time there in proximity to the active ultrasonic speaker. In contrast, SAL-treated rats, which had received

SAL also before the first test, did not display a clear social approach response. Although the number of proximal zone entries increased in response to 50-kHz USV playback, the time spent in proximity to the active ultrasonic speaker was not significantly higher than before playback, indicative of the habituation phenomenon in rats repeatedly exposed to SAL. Because both groups received SAL immediately before the retest, the difference between the two groups in the retest is driven by their unique experiences made during the first test, most notably changes in the subject's internal state systematically manipulated through pharmacological treatment. This shows that the observed behavioral effects are not simply acute drug effects because both groups received SAL immediately before the retest. Rather, this may indicate that the subject's internal state during the first test can affect the outcome of the retest. Specifically, the lack of the habituation phenomenon in rats exposed to HALO before the first test but treated with SAL before the retest appears to be due to differences in the subject's internal state between the first test and retest. Pharmacological treatment, including the administration of HALO, has profound effects on the subject's internal state, e.g., as reflected by its effect on reward expectation (Negrelli et al., 2020) and the emission of 50-kHz USV (Wright et al., 2013), and it was repeatedly shown that a mismatch of the subject's internal state during acquisition, i.e., the first test, and retrieval, i.e., the retest, hinders memory retrieval (e.g., Girden and Culler, 1937; for review see Radulovic et al., 2017). For instance, in a T-maze escape task, rats were not able to display a response learned under drugged conditions in later undrugged conditions (Overton, 1964). Alternatively, lack of the habituation phenomenon might be due to the HALO-dependent prevention of approach experience in the first test. As compared to the other two experiments, however, the result pattern is less clear and SAL-treated rats did not differ from each other dependent on whether they received SAL or HALO before. Replication experiments with a higher number of subjects per experimental group are thus warranted. The two groups which had received HALO prior to the retest did not display social approach responses at all, which might reflect a floor effect, namely the combination of habituation due to repeated 50-kHz USV playback experience and the drug's inhibitory effect on locomotor activity, presumably completely blocking the mismatch-induced recurrence of the social approach response in the rats receiving SAL before the first test and HALO before the retest. Of note, the fact that the habituation phenomenon is not only evident in a radial maze but also occurs in a different setup, i.e., a platform, speaks against the idea that the habituation phenomenon is driven by aspects specific to the radial maze.

Finally, we repeatedly reported the occurrence of response calls emitted by the rats exposed to playback of 50-kHz USV (Wöhr and Schwarting, 2007, 2009; Willadsen et al., 2014; Willuhn et al., 2014; Engelhardt et al., 2017, 2018; Berg et al., 2018; Kisko et al., 2020). Here, we now show that the habituation phenomenon does not only occur at the level of the social approach response but also includes the emission of response calls. While the majority of the rats emitted response calls when exposed to playback of 50-kHz USV during the first test, a week later only a small minority emitted response calls during the retest. The habituation at the level of the response calls was so prominent that it occurred independently of whether drug treatment was changed between the first test and the retest in all groups. Importantly, no rat emitted response calls during playback of noise, indicating that the emission of response calls is specifically induced by playback of 50-kHz USV. Future studies on the functional significance of such response calls appear warranted.

Conclusions

In the present series of experiments, we showed that the habituation phenomenon displayed by rats in response to repeated playback of 50-kHz USV is characterized by limited generalizability because it is present in Wistar but not Sprague-Dawley rats. While this is surprising considering a similar phenomenon in mice, this might offer a tool to identify relevant differences that underlie the habituation phenomenon. We further demonstrated that the habituation phenomenon in Wistar rats can be overcome by AMPH treatment, presumably by boosting meso-limbic DA signaling previously shown to be involved in closing a perception-to-action-loop through linking mechanisms relevant for the detection of 50-kHz USV to behavioral responses, such as social approach. Finally, we revealed that the habituation phenomenon is dependent on the subject's internal state. State-dependency indicates that impaired memory retrieval due to a mismatch of the subject's internal states between first and second exposure to 50-kHz USV contributes to the habituation phenomenon. This is in line with previous evidence emphasizing the importance of intact memory function.

Limitations of study

While the present series of experiments provide important insights into the habituation phenomenon in rats by demonstrating limited generalizability, pharmacological modulation, and state-dependency of the habituation toward pro-social 50-kHz calls, there are limitations. First, additional experiments are warranted in order to better understand the role of anxiety and stress and whether the habituation phenomenon depends on how anxiogenic the environment is. Second, because the pharmacological modulation of the habituation phenomenon through AMPH might be at least partly driven by an unspecific stimulatory effect, it appears relevant to assess the consequences of other catecholaminergic compounds. Finally, replication experiments with a higher number of subjects per experimental group would be beneficial, ideally including aggregated preference measures for comparing experimental conditions.

Resource availability

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Markus Wöhr (markus.wohr@kuleuven.be).

Material availability

This study did not generate new unique reagents.

Data and code availability

All data supporting the results can be found in this manuscript. Data requests can be addressed to the corresponding author.

METHODS

All methods can be found in the accompanying transparent methods supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2021.102426.

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AUTHOR CONTRIBUTIONS

RS and MW designed the study and acquired resources and funding. AB performed the experiments. AB with substantial help from CPS and MW analyzed the data. AB, RS, and MW wrote the manuscript with substantial contributions from CPS. RS and MW oversaw the project.

DECLARATION OF INTERESTS

Markus Wöhr is scientific advisor of Avisoft Bioacoustics. The other authors declare no competing interests.

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Supplemental information

Limited generalizability, pharmacological modulation, and state-dependency of habituation towards pro-social 50-kHz calls in rats

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Limited generalizability, pharmacological modulation, and state-dependency of the habituation towards pro-social 50-kHz calls in rats

Original Research Article

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Supplementary Material

Transparent Methods

Overview

Three separate playback experiments were conducted in juvenile male rats. In Experiment 1, we compared the approach outcomes of 50-kHz USV (or noise) between Wistar and Sprague-Dawley rats during a first test and a retest conducted one week later (2 groups: Wistar, Sprague-Dawley, N=18 subjects each). In Experiment 2, we tested the effects of systemic AMPH (versus SAL) injected prior to the retest (2 groups: AMPH, SAL, N=10 subjects each), and in Experiment 3, we tested how HALO injected prior to the first test and/or retest affects approach (4 groups, first test - retest: SAL-SAL, HALO-SAL, SAL-HALO, HALO-HALO, N=12 subjects each). In each experiment, we also measured locomotor activity to test for psychomotor effects regarding stock (Experiment 1) or drug treatment (Experiments 2 and 3).

Animals and Housing

In all playback experiments, we used juvenile male rats obtained from Charles-River, Germany, at an age of about 6 weeks.

Experiment 1: Thirty-six rats (N=18 Wistar, N=18 Sprague-Dawley) were used, weighing 163.47±2.85 g (range 138.5-205.0 g) at the time of the first test, and 224.68±3.34 g (range 189.5-267.0 g) in the retest one week later.

Experiment 2: Twenty Wistar rats were used, which weighed 144.25±1.88 g (range 128.5-164.5 g) at the time of the first test, and 206.81±2.58 g (range 177.5-230.5 g) in the retest one week later. Based on their approach responses to 50-kHz USV playback in the first test, they were pseudo-randomized and split into two groups (n=10 each) with similar performance in the first test.

Experiment 3: Forty-eight male Wistar rats were used, which weighed 189.57±2.94 g (range 147.5-233.0 g) at the time of the first test, and 248.61 ± 2.97 g (range 210.0-293.5 g) in the retest one week later. They were assigned randomly to four treatment groups: SAL-SAL, HALO-SAL, SAL-HALO, HALO-HALO.

In each experiment, we used standard housing conditions and groups of 5-6 rats per cage (polycarbonate, macrolon type IV, size 380x200x590 mm with high steel covers), with water and food available *ad libitum*, a 12/12 light-dark cycle with lights on at 7 am, and humidity ranging between 32-50 %. After arrival from the breeder, rats had seven days of acclimatization, followed by a standard protocol of handling on three consecutive days (five minutes each). All procedures had been approved by the ethical committee of the local government (Regierungspräsidium Gießen, Germany, TVA Nr. 35-2018).

Drugs

D-AMPH sulfate (Sigma, MO, USA) was dissolved in 0.9 % SAL and administered intraperitoneally (i.p.) at a volume of 2 ml/kg and a dose of 2.5 mg/kg (expressed as salt) based on the findings by Engelhardt et al. (2017). Immediately after injection of AMPH or SAL, the given rat was placed on the radial maze.

According to Tonelli et al. (2018a), HALO (0.5 mg/kg; Haldol, Janssen, Belgium) or SAL was injected intraperitoneally (i.p.) 60 min before placing the rat on the testing platform. During that pre-testing period, the animal was kept in a dark room in a single cage with fresh bedding. A dose of 0.5 mg/kg HALO was previously reported to block almost 100 % of all striatal DA D2 receptors (Kapur et al., 2000).

Acoustic Stimuli and Experimental Setups

Two types of stimuli were used: A) 50-kHz USV, which had been recorded from an adult male Wistar rat (ca. 350 g) during exploration of a cage containing scents from a recently separated cage mate (for details see Wöhr et al., 2008). This stimulus material was composed of a sequence lasting 3.5 s, presented in a loop. Each sequence contained 13 50-kHz calls (total calling time: 0.90 s), with 10 of them being frequency-modulated and 3 flat (for details see Wöhr and Schwarting, 2007; Figure 1A). As in previous studies, peak amplitude was about 70 dB (measured from a distance of 40 cm), which is within the typical range of 50-kHz USV (Kisko et al., 2018; 2020). B) Noise: This artificial time- and amplitude-matched noise was generated with SASLab Pro (Version 4.2, Avisoft Bioacoustics, Germany; for details see Wöhr and Schwarting, 2007; Figure 1B). Specifically, each given 50-kHz USV in the original natural 50-kHz USV stimulus material was replaced by noise with durations and amplitude modulations matching to those of the original 50-kHz USV. Thus, the stimulus series had the same temporal patterning and was identical to the original natural 50-kHz USV series with respect to all call features, apart from the fact that sound energy was not confined to a certain frequency as in case of the natural 50-kHz USV.

The acoustic stimuli were presented through an ultrasonic loudspeaker (ScanSpeak, Avisoft Bioacoustics), which had a frequency range of 1-120 kHz with flat frequency response (+/- 12 dB) between 15 and 80 kHz. Sounds were played via an external sound card with a sampling rate of 192 kHz (Fire Wire Audio Capture FA-101, Edirol, London, UK) and a portable ultrasonic power amplifier with a frequency range of 1-125 kHz (Avisoft Bioacoustics).

<u>Radial maze playback paradigm (Experiments 1 and 2):</u> Social approach induced by 50-kHz USV was assessed on a radial eight-arm maze (arms 40.5 x 9.8 cm; Figure 1C, left), elevated 52 cm above the floor, as described by Wöhr and Schwarting (2007), which was monitored by a Basler aca camera placed 150 cm centrally above the radial maze. The ultrasonic speaker used for stimulus presentation was placed 20 cm away from the end of one arm and an additional, but inactive speaker was arranged symmetrically at the opposite arm as a visual control. For testing, the given rat was placed into the center of the maze, facing away from both ultrasonic speakers. After an initial 15-min habituation period, it was exposed to 5-min playback presentations of 50-kHz USV and noise, separated by a 10-min inter-stimulus interval. Acoustic stimulus presentations (50-kHz USV, noise) were ordered in a counterbalanced manner, which was the same in the first test and retest. The session ended after an additional 10-min post-stimulus phase (Figure 1D).

<u>Platform playback paradigm (Experiment 3):</u> A squared platform of 100 x 100 cm (Figure 1C, right), elevated 50 cm above the floor, was used, as described by Tonelli et al. (2018a). Approximately 150 cm above the platform, a Basler aca camera was placed centrally above the platform. The speakers were placed symmetrically at opposite sides, 20 cm away from the platform. One served as the active speaker and the other as a visual control. For testing, a given rat was placed in the center of the squared platform, facing away from both ultrasonic speakers. After an initial 15-min habituation period, it was exposed to 5-min playback presentations of 50-kHz USV or noise, separated by a 10-min inter-stimulus interval, as for the radial maze playback paradigm (Figure 1D).

In both paradigms, playback and possible calls of the tested subject were monitored with two ultrasonic condenser microphones (CM16, Avisoft Bioacoustics) placed next to the loudspeakers. Testing took place under red light (~10 lux), with no other rats present in the testing room and between 7-17 h. Prior to each test the equipment was cleaned thoroughly with acetic acid 0.1 % and dried afterwards.

Overt Behavior and Analysis

Behavior was recorded via the video camera and analyzed using EthoVison XT (Version 13, Noldus, The Netherlands). Locomotor activity was measured in terms of distance traveled and was expressed in cm. In Experiments 1 and 2, the numbers of entries into the three arms proximal and the three arms distal to the active ultrasonic loudspeaker and the times spent thereon were quantified (Figure 1E, left). Proximal measures served for stimulus-directed activity, i.e. approach to 50-kHz USV playback, as in previous studies (e.g. Seffer et al., 2014). To quantify approach in Experiment 3, the platform was virtually divided into 25 quadrants (each 20 x 20 cm). The six quadrants lateral to the active speaker were defined as the proximal zone and those lateral to the control speaker were defined as the distal zone (Figure 1E, right). Entries into and times spent within these zones served for quantification of approach to 50-kHz USV playback, as in previous studies (e.g. Tonelli et al., 2018a). Heat maps were generated using EthoVison XT (Noldus) and show the average of each experimental group during specific time windows.

USV Recording and Analysis

The ultrasonic microphones were connected via an UltraSoundGate 416H USB audio device (Avisoft Bioacoustics) to a PC, where acoustic data were recorded with a sampling rate of 250 kHz (16-bit format; recording range 0-125 kHz) by RECORDER USGH (Avisoft Bioacoustics). For USV analysis, recordings were converted into high-resolution spectrograms (frequency resolution 488 Hz; time resolution 0.512 ms) via fast Fourier transformation (512 FFT length, 100 % frame, Hamming window, and 75 % time-window overlap) using SASLab Pro software 5.2.09 (Avisoft Bioacoustics). Calls with frequencies higher than 33 kHz were defined as 50-kHz USV and were counted by a trained observer who was blind with respect to group assignment. The following acoustic features were determined, as described previously (Kisko et al., 2018): call duration, peak frequency, and frequency modulation. Peak frequency was 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.

Statistical Analysis

Locomotor activity: To test whether general locomotor activity differed between groups, ANOVAs for repeated measures with the within-subject factor time (first 15 min of a given test) and between-subject factor group (i.e. rat stock or drug treatment) were calculated. To compare locomotor activity between groups at individual time points (single minutes), two-tailed *t*-tests for independent samples were used. In Experiment 3, locomotor activity was compared between the first test and the retest using a two-tailed paired *t*-test. Differences within groups were tested post-hoc with *t*-tests or LSD tests whenever appropriate.

Response to playback: According to Wöhr and Schwarting (2012), the responses to playback were expressed as change scores, which were calculated by subtracting entry or time measures proximal or distal to the acoustic source during the 5 min before stimulus presentation from those during the 5 min of stimulus presentation. To test for stimulus effects, these scores were compared with one-sample *t*-tests (versus 0). Paired *t*-tests were used for comparing proximal versus distal changes in arm entries or times spent thereon. In the first playback test, we used one-tailed *t*-tests, since we had repeatedly shown that rats display a preference for proximal arms during 50-kHz USV playback (e.g. Wöhr and Schwarting, 2007). In the retest, two-tailed *t*-tests were used, because no assumptions could be made relying on earlier experiments. When the change values of the first test and retest were compared, two-tailed paired *t*-tests were applied. For comparing experimental groups, ANOVAs with the between-subject factors drug treatment before the first test and retest or two-tailed unpaired *t*-tests were used. To assess stimulus-directed activity with high temporal resolution, ANOVAs for repeated measures with the within-subject factor time (5 min playback phase) and preference (proximal versus distal) were calculated, followed by paired *t*-tests for single minutes when appropriate. The number of rats emitting response calls was compared between experimental groups using the chi^2-test.

Data are presented as means±SEM (standard error of the mean). Average heat maps reflect actual dwell times and not change scores. A p-value of <0.050 was considered statistically significant.

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Response Calls Evoked by Playback of Natural 50-kHz Ultrasonic Vocalizations in Rats

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Rats are highly social animals known to communicate with ultrasonic vocalizations (USV) of different frequencies. Calls around 50 kHz are thought to represent a positive affective state, whereas calls around 22 kHz are believed to serve as alarm or distress calls. During playback of natural 50-kHz USV, rats show a reliable and strong social approach response toward the sound source. While this response has been studied in great detail in numerous publications, little is known about the emission of USV in response to natural 50-kHz USV playback. To close this gap, we capitalized on three data sets previously obtained and analyzed USV evoked by natural 50-kHz USV playback in male juvenile rats. We compared different rat stocks, namely Wistar (WI) and Sprague-Dawley (SD) and investigated the pharmacological treatment with the dopaminergic D2 receptor antagonist haloperidol. These response calls were found to vary broadly inter-individually in numbers, mean peak frequencies, durations and frequency modulations. Despite the large variability, the results showed no major differences between experimental conditions regarding call likelihood or call parameters, representing a robust phenomenon. However, most response calls had clearly lower frequencies and were longer than typical 50-kHz calls, i.e., around 30 kHz and lasting generally around 0.3 s. These calls resemble aversive 22-kHz USV of adult rats but were of higher frequencies and shorter durations. Moreover, blockade of dopamine D2 receptors did not substantially affect the emission of response calls suggesting that they are not dependent on the D2 receptor function. Taken together, this study provides a detailed analysis of response calls toward playback of 50-kHz USV in juvenile WI and SD rats. This includes calls representing 50-kHz USV, but mostly calls with lower frequencies that are not clearly categorizable within the so far known two main groups of USV in adult rats. We discuss the possible functions of these response calls addressing their communicative functions like contact or appeasing calls, and whether they may reflect a state of frustration. In future studies, response calls might also serve as a new read-out in rat models for neuropsychiatric disorders, where acoustic communication is impaired, such as autism spectrum disorder.

Keywords: ultrasonic vocalizations, animal communication, playback, stock, strain, haloperidol, Wistar, Sprague-Dawley

INTRODUCTION

Acoustic communication among conspecifics is an important aspect of the social life of many species and often essential for maintaining stable social structures. A characteristic feature of acoustic communication in several species is its reciprocal nature where a signal emitted by the sender frequently evokes the emission of a response signal in the receiver (Seyfarth and Cheney, 2003).

Many rodent species communicate through so-called ultrasonic vocalizations (USV), i.e., within frequencies not audible for humans (Brudzynski, 2010). In juvenile and adult rats, two main types of vocalizations are typically distinguished (Brudzynski, 2013a; Wöhr and Schwarting, 2013). Vocalizations with frequencies around 22 kHz are referred to as aversive or distress calls, presumably representing a negative affective state (Blanchard et al., 1991; Fendt et al., 2018). Vocalizations with frequencies around 50 kHz are thought to represent a positive affective state usually emitted during appetitive situations like play or mating (Knutson et al., 1998; Panksepp, 2005). These appetitive calls are typically characterized by frequencies between 35 and 80 kHz and durations in a range of 10–150 ms (Burgdorf et al., 2008; Wöhr et al., 2008; Takahashi et al., 2010). Often, such 50-kHz USV are categorized and the call categories flat, step, trill, and mixed are commonly differentiated (Kisko et al., 2018). Aversive 22-kHz USV, in contrast, have been defined between frequencies of 18 and 32 kHz (Brudzynski, 2001) and within this frequency range, short (<300 ms) and long (>300 ms) calls were identified (Brudzynski et al., 1993). Long 22-kHz calls were found to be emitted during situations of external danger, such as during the presence of a predator or during predator odor exposure, and are usually associated with freezing behavior (Blanchard et al., 1991; Fendt et al., 2018; Simmons et al., 2018). Short 22-kHz USV, however, are much more ambiguous and their function has not been identified yet (Brudzynski, 2021). It was suggested that short 22-kHz USV represent internal distress without external influence, like frustration (Taylor et al., 2019). In addition, they were repeatedly reported to occur during drug withdrawal (Ma et al., 2010; Simmons et al., 2018).

The communicative functions of 22- and 50-kHz USV can be studied by means of playback experiments (Seffer et al., 2014) and it was shown that they elicit distinct behavioral responses pattern in the receiver (Wöhr et al., 2016). Playback of natural 22-kHz USV usually induces a defensive response, including avoidance behavior and behavioral inhibition (Brudzynski and Chiu, 1995; Fendt et al., 2018). Playback of natural 50-kHz USV, in contrast, evokes social approach behavior toward the sound source (Wöhr and Schwarting, 2007). At the physiological level, playback of 22- and 50-kHz USV entail to distinct alterations. While playback of 22-kHz leads to a decrease in heart rate during behavioral inhibition, heart rate is increased during social approach behavior in response to playback of 50-kHz USV (Olszyński et al., 2020). Likewise, distinct brain activation patterns are observed. Playback of 22-kHz USV induces increased activity in the amygdala (Sadananda et al., 2008; Parsana et al., 2012), whereas playback of 50-kHz USV results in an activation of the nucleus accumbens (Sadananda et al., 2008), where it causes a phasic release of dopamine (Willuhn et al., 2014).

At the behavioral level, the social approach response toward 50-kHz USV playback can be accompanied by the emission of response calls (Wöhr and Schwarting, 2007, 2009; Willadsen et al., 2014; Willuhn et al., 2014; Engelhardt et al., 2017, 2018; Berg et al., 2018, 2021; Kisko et al., 2020; Olszyński et al., 2020, 2021). Although echoing the reciprocal nature of acoustic communication and repeatedly observed in studies applying the 50-kHz USV playback paradigm, still little is known about such response calls. In previous studies, response calls toward 50-kHz USV were observed in males and females (Berg et al., 2018, 2021), albeit the emission of calls in response to 50-kHz USV playback was found to be more prominent in males than females in one study (Kisko et al., 2020). A developmental study further suggests that age is another relevant factor, with juvenile rats emitting more response calls than adult rats (Wöhr and Schwarting, 2009). Finally, prior experiences (Olszyński et al., 2021) and inter-individual differences (Engelhardt et al., 2018) were also reported to play a role. However, the function of response calls remains elusive, which is why we wanted to shed light onto the meaning and the importance of response calls in social situations like the 50-kHz USV playback.

To close this gap, we capitalized on a previously obtained large data set and analyzed USV evoked by natural 50-kHz USV playback in male juvenile rats (Berz et al., 2021). In our previous study, we showed, amongst others, that the social approach response toward 50-kHz calls is a stable phenomenon that occurs in Wistar (WI) and Sprague-Dawley (SD) rats and that it can be modulated by administration of the dopaminergic D2 receptor antagonist haloperidol (Halo; Berz et al., 2021). Here, we present three new data sets from these previous experiments. Data set 1 was comprised of WI rats exposed to 50-kHz USV playback. We analyzed it in an initial attempt to better understand the emission of response calls and to test whether response calls occur specifically in reaction toward 50-kHz USV but not noise and whether stimulus order of 50-kHz USV and noise plays a role. Data set 2 consisted of WI and SD rats and their response calls were compared to see whether there was a difference between the stocks. In the final data set 3, rats received either Halo or saline (Sal) to investigate whether Halo treatment not only affects social approach behavior but also the emission of response calls toward 50-kHz USV playback. Our comprehensive analysis approach included a detailed investigation of the temporal emission pattern and an examination of acoustic features, focusing on numbers of calls, latencies to start calling, mean peak frequencies, call durations, and frequency modulations.

MATERIALS AND METHODS

Animals and Housing

In total, 108 experimentally naïve juvenile male rats around 5–7 weeks of age (Charles River Laboratories, Sulzfeld, Germany) were analyzed. The sample consisted of 90 Wistar (WI) wildtype rats and 18 Sprague-Dawley (SD) wildtype rats. The animals were kept in a vivarium with a 12-hour light/dark cycle with lights on at 7 am and 32–50% humidity. They were housed in groups of five to six rats in polycarbonate cages (macrolon type IV, size 380 × 200 × 590 mm with high steel covers) where food and water were provided *ad libitum*. After arrival from the breeder, the animals had seven days to acclimate to the vivarium, followed by a standardized protocol of handling for three consecutive days, each day for 5 min. The procedures had been approved by the ethical committee of the local government (Regierungspräsidium Gießen, Germany, TVA Nr. 6 35-2018).

Overview

Response calls were analyzed in three data sets. These sets were obtained as part of a recently published study focusing on the habituation of the social approach response to repeated playback of 50-kHz USV (Berz et al., 2021). In this previous study, rats were exposed twice to playback of 50-kHz USV and their behavioral response was quantified, i.e., locomotor activity and approach behavior. Here, we now analyzed response calls evoked by playback of 50-kHz USV that were also recorded in this study. We focused on the emission of response calls during the first playback exposure because preliminary data indicate that call emission decreases with repeated playback presentations similar to social approach behavior (Berz et al., 2021). In the first data set, we analyzed response calls in WI rats ($N = 24$) and tested whether their emission occurs specifically during playback of 50-kHz USV but not noise and whether their emission depends on stimulus order. Rats were weighing 144.25 ± 1.88 g (range 128.5–164.5 g). In the second data set, we compared the production of response calls between WI rats ($N = 18$) to that of SD rats ($N = 18$). Rats were weighing 163.47 ± 2.85 g (range 138.5–205 g). In the third data set, we studied the role of the dopaminergic system in regulating the emission of response calls and compared response calls emitted by WI rats systemically treated with the dopaminergic D2 receptor antagonist Halo ($N = 24$) and saline treated controls ($N = 24$). Rats were weighing 189.57 ± 2.95 g (range 147.5–233 g).

Drug Treatment

In the third data set, rats received the dopaminergic D2 receptor antagonist Halo (0.5 mg/kg; Haldol, Janssen, Belgium) or saline (Sal, 0.9% NaCl solution, Braun, Germany). The ip injection took place 60 min before the start of the playback experiment and during the time between the injection and the playback experiment, rats were kept singly (in a small cage with bedding and water *ad libitum*) in a dark room (according to Tonelli et al., 2017).

50-kHz Ultrasonic Vocalizations Playback: Setup

As experimental setups, an eight-arm radial maze (data sets 1 and 2) and a squared platform (data set 3), each elevated 52 cm above the ground, were employed. On two opposite sides of the given apparatus, an ultrasonic speaker (ScanSpeak, Avisoft Bioacoustics, Berlin, Germany) and an ultrasonic condenser microphone (CM16, Avisoft Bioacoustics) were placed 20 cm away from the end of the arm or platform. Only one of the speakers was active, whereas the other one served as a visual control. Experiments were conducted under red light (∼10 lux).

50-kHz Ultrasonic Vocalizations Playback: Acoustic Stimuli

We presented two types of acoustic stimuli: (A) 50-kHz USV recorded from an adult male WI rat (ca. 350 g) during exploration of a cage containing scents from a recently removed cage mate (for details see Wöhr et al., 2008). This recording was composed of a sequence of 3.5 s with 13 different 50-kHz calls (total calling time 0.9 s) presented in a loop (for details see Wöhr and Schwarting, 2007). The peak amplitude was 70 dB (measured from a distance of 40 cm), being in the typical range of 50-kHz USV (Kisko et al., 2020). (B) Time- and amplitude-matched noise was generated with SASLab Pro (Version 4.2, Avisoft Bioacoustics) by replacing each 50-kHz call by noise with matching duration and amplitude modulation. Accordingly, each noise playback series had the same temporal pattern and all call features were identical, except that the sound energy was not in a certain frequency range as in the natural 50-kHz USV playback (for details see Wöhr and Schwarting, 2012). The acoustic stimuli were presented *via* an ultrasonic speaker (ScanSpeak, Avisoft Biosacoustics) with a frequency range of 1–120 kHz and a flat frequency response (±12 dB) between 15 and 80 kHz. Sounds were played *via* a portable ultrasonic power amplifier with a frequency range of 1–125 kHz (Avisoft Bioacoustics) and *via* an external sound card with a sampling rate of 192 kHz (Fire Wire Audio Capture FA-101, Edirol, London, United Kingdom).

50-kHz Ultrasonic Vocalizations Playback: Paradigm

At the beginning of the playback experiment, rats were placed individually in the center of the eight-arm radial maze (data sets 1 and 2) or the squared platform (data set 3). After an initial habituation period of 15 min, the first playback presentation of 5 min duration commenced. The second playback presentation of 5 min duration followed after an inter-stimulus interval of 10 min. Acoustic stimuli (i.e., 50-kHz USV, noise) were presented in a counterbalanced manner. The trial ended with a post-stimulus interval of 10 min. The whole paradigm lasted 45 min.

Recording and Analysis of Response Calls

For recording response calls emitted by the given experimental rat, two ultrasonic microphones were placed symmetrically on two sides of the maze (data sets 1 and 2) or the platform (data set 3) next to the speakers. They were connected *via* an UltraSoundGate 416H USB audio device (Avisoft Bioacoustics) to a computer, where acoustic data were recorded with a sampling rate of 250 kHz (16-bit format; recording range 0–125 kHz) using RECORDER USGH (Avisoft Bioacoustics). For acoustical analysis, recordings were transferred to DeepSqueak (version 2.6.1, Windows standalone), a deep learning-based system for detection and analysis of USV (Coffey et al., 2019). Recorded files were converted into high-resolution spectrograms and were analyzed using the pre-trained automated "short rat call network

V2." The settings for call detection were "high recall," with an overlap of 0.001 s. This setting was chosen because it minimizes the possibility that a call is missed, albeit at the cost of false positives by including noise. Therefore, a custom trained network for denoising was applied afterward. The detected events were then transferred into the DeepSqueak Screener (Fork on GitHub by L. Lara-Valderrábano and R. Ciszek: 10.5281/zenodo.3690137),[1] where the files were reviewed and denoised again manually by an experienced observer accepting (response calls) or rejecting (noise or playback calls) events. All response calls, irrespective of frequencies and durations, were counted. For later analysis, response calls during the 5 min before, during, and after the playback presentations (50-kHz USV or noise) were taken into account (referred to as stimulus phase). Outside this time window, calls occurred rarely. Acoustic features, i.e., call duration, peak frequency, and frequency modulation (difference between highest and lowest frequency), were defined and analyzed as described previously (Kisko et al., 2018). For classifying response calls, we applied previously established frequency thresholds (Brudzynski, 2001). Calls with frequencies higher than 32 kHz were classified as 50-kHz USV and calls below 32 kHz were defined as 22-kHz USV.

Recording and Analysis of Overt Behavior

As pointed out above, the behavioral data (locomotion, approach) were part of a recently published study focusing on the habituation of the social approach response to playback of 50-kHz USV (Berz et al., 2021). Here, we reconsidered these data in the context of the new data on response calls in order to address the question whether locomotor activity and approach behavior evoked by playback of 50-kHz USV are associated with the emission of response calls. Briefly, overt behavior was recorded and analyzed using EthoVision XT (Version 13, Noldus, The Netherlands). Locomotion was measured by the distance traveled. For quantifying approach behavior on the maze (data sets 1 and 2), the numbers of entries into the three arms proximal and distal to the active speaker and the time spent thereon were measured. For quantifying approach behavior on the platform (data set 3), it was virtually divided into 25 equal quadrants, with the six quadrants close to the active speaker serving as proximal zone, while the six quadrants close to the inactive speaker were defined as distal zones. Entries and time spent in these zones were measured (for details see Berz et al., 2021).

Statistical Analysis

Analyses of variance (ANOVAs) for repeated measurements were calculated with the between-subject factors playback order (50-kHz USV first vs. second), stocks (WI vs. SD), or drug treatment (Halo vs. Sal), and the within-subject factors stimulus phase (5 min before, during, or after playback) and playback stimulus (50-kHz USV or time- and amplitude-matched noise). This was followed by two-tailed *t*-tests for comparing individual experimental groups. The ratio between calling and non-calling rats was evaluated by a χ^2-test (calculated using https://www.socscistatistics.com/tests/chisquare2/default2.aspx). Approach behavior was quantified by subtracting the times spent on proximal arms (or in proximal zones) before the 5 min of 50-kHz USV playback from the time spent there during the 5 min of playback. The same was done with the entries into proximal arms or zones. Pearson correlation coefficients (bivariate) were calculated for the correlation between numbers of emitted calls and approach behavior. For testing a possible correlation with locomotor behavior, locomotion (distance traveled in cm) during the 5 min before playback were subtracted from that during the 5 min during playback. This number was then correlated with the numbers of response calls emitted using the Pearson correlation coefficient. For general locomotor activity correlations, the distance traveled during the initial 15-min habituation period were taken into account. All *t*-tests, ANOVAs, and correlations were calculated with IBM SPSS Statistics (version 25). Graphs were made using GraphPad Prism (version 8). Data are represented as means ± SEM (standard error of mean). A *p*-value of < 0.050 was considered statistically significant.

RESULTS

Data Set 1: Response Calls

Call Numbers and Latencies

Playback of 50-kHz USV induced response calls in the majority of WI rats. Among the 24 rats of data set 1, 23 of them emitted response calls. The mean number of response calls was 123.5 ± 26.21, ranging between 0 and 414 calls in total per rat (**Figure 1A**). During the 5 min before 50-kHz USV playback, no calls were emitted. The occurrence of response calls was not dependent on whether 50-kHz USV were presented as the first or the second stimulus ($t_{22} = 0.82$, $p = 0.21$). Importantly, high levels of response calls were emitted specifically in reaction toward playback of 50-kHz USV but not noise, irrespective of whether 50-kHz USV were presented as the first ($t_{11} = 2.8$, $p = 0.017$) or the second stimulus ($t_{11} = 4.013$, $p = 0.002$; **Figure 1A**). The latency to start calling after onset of 50-kHz USV was 20.17 ± 88.17 s (**Figure 1B**). Stimulus order did not affect call latency ($t_{21} = 0.52$, $p = 0.61$). Therefore, we abstained from differentially considering stimulus order further in all following analyses.

Data Set 2: Stock Differences

Call Numbers and Latencies

Consistent with data set 1, response calls were seen in the majority of rats in data set 2 focusing on possible stock differences between WI and SD rats. From the two different stocks, 10 out of the 18 WI rats emitted calls in response to 50-kHz USV playback and 12 out of 18 SD rats did. The ratios between calling and non-calling rats did not differ between stocks ($x^2{}_{1,\ 36} = 0.468$, $p = 0.49$). Likewise, the mean numbers of response calls (**Figure 2A**; $t_{34} = 0.032$, $p = 0.975$; WI: 44.39 ± 17.81; SD: 45.17 ± 16.45) as well as the latencies to start calling (**Figure 2B**; $t_{20} = 0.547$, $p = 0.590$; WI: 50.56 ± 41.16 s; SD: 29.88 ± 4.93 s) did not differ between WI and SD. In both stocks, high levels of response calls were exclusively evoked by playback of 50-kHz USV, while response calls rarely

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[1] https://github.com/UEFepilepsyAIVI/DeepSqueak.git

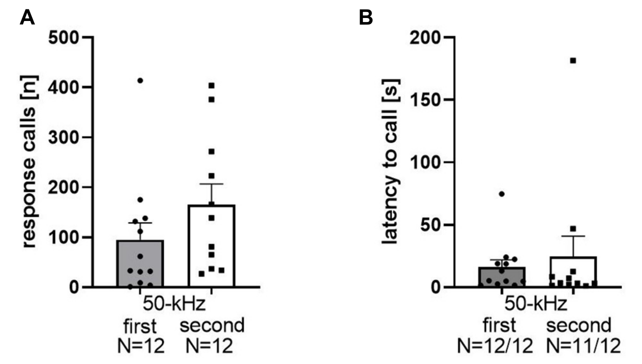

FIGURE 1 | Total numbers of response calls emitted **(A)** for playback of 50-kHz as the first stimulus [95.25 ± 33.62 (mean ± SEM)] or as the second stimulus (152 ± 40.07) of WI rats. Latencies after stimulus onset **(B)**: 50-kHz first: 16.04 ± 5.67; 50-kHz second: 24.67 ± 16.19.

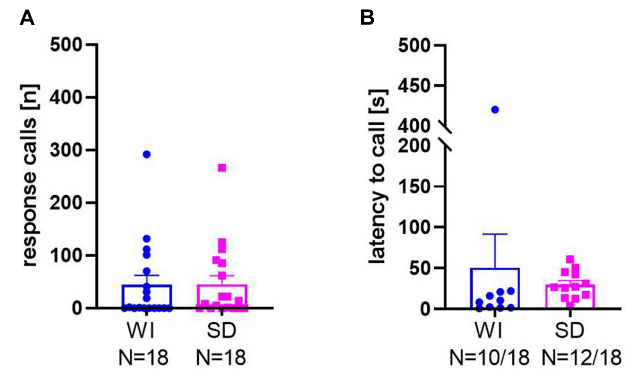

FIGURE 2 | Total numbers of response calls **(A)** and latencies to call **(B)** in Wistar (WI) and Sprague-Dawley (SD) rats.

occurred during noise playback (WI: $t_{17} = 2.717$, $p = 0.015$; SD: $t_{17} = 2.727$, $p = 0.014$).

Data Set 1 and 2: Detailed Analyses

Temporal Emission Pattern

We next pooled the data sets 1 and 2 and performed more detailed analyses. First, a detailed temporal analysis revealed that the emission of response calls was strongly dependent on stimulus ($F_{1,\ 58} = 21.260$, $p < 0.001$) and stimulus phase ($F_{2,\ 116} = 21.120$, $p < 0.001$), with an interaction between stimulus and stimulus phase ($F_{2,\ 116} = 21.002$, $p < 0.001$), while stock had no major impact (stock: $F_{1,\ 58} = 2.311$, $p = 0.134$; stock × stimulus: $F_{1,\ 58} = 2.253$, $p = 0.139$; stock × stimulus phase: $F_{2,\ 116} = 2.308$, $p = 0.104$; stock × stimulus × stimulus phase: $F_{1,\ 116} = 2.290$, $p = 0.106$; **Figure 3**). Specifically, playback of 50-kHz USV but not noise led to a prominent increase in response calls, which occurred during the 5 min of 50-kHz USV playback and up to 5 min thereafter. The peak of vocalization typically occurred in the second or third minute after 50-kHz USV playback onset. With onset of the 50-kHz USV playback, the numbers of emitted response calls increased significantly in WI ($F_{1,\ 41} = 27.940$, $p < 0.001$) and SD rats ($F_{1,\ 17} = 7.436$, $p = 0.014$). After that, calling rate decreased to zero at the latest 5 min after the playback had ended. In both stocks, substantial calling only occurred in response to 50-kHz USV playback and not in response to noise, reflecting high specificity of response call emission (WI: $F_{1,\ 41} = 25.387$, $p < 0.001$; SD: $F_{1,\ 17} = 7.538$, $p = 0.014$). Furthermore, the call emission sequence showed that most animals started calling with higher frequencies around 50 kHz and quickly changed to emit calls of frequencies around 22 kHz (**Supplementary Figure 1A**).

Response Call Features

Secondly, detailed analyses of acoustic features revealed that the calls in response to 50-kHz USV playback were heterogeneous since they were characterized by a large variability in acoustic features and shapes. Both, WI and SD rats emitted calls below and above 32 kHz. These calls had rather different durations and shapes, and the temporal spaces between them varied substantially.

For a further quantification of the response calls, mean peak frequencies, mean call durations, and mean frequency modulations were quantified (**Figure 4**; for examples of response calls, see **Figure 5**). Peak frequencies of WI rats (32.48 ± 1.46 kHz) and SD rats (37.82 ± 3.2 kHz; **Figure 4A**) did not differ significantly from each other ($t_{15{\cdot}82} = 1.52$, $p = 0.149$). Call durations of WI rats (0.34 ± 0.03 s) tended to be longer than those of SD rats (0.24 ± 0.05 s; $t_{43} = 1.859$, $p = 0.07$). Frequency modulations did not differ between stocks ($t_{43} = 0.98$, $p = 0.33$; WI: 6.68 ± 0.51 kHz; SD: 7.68 ± 0.97 kHz).

To visualize the different call parameters and the distribution of individual calls, scatter plots for either call durations or frequency modulations were plotted vs. peak frequencies (**Figure 6**). This analysis showed that most calls were below 32 kHz, with durations above and below 0.3 s. Frequency modulations were mainly below 5 kHz. The main distribution of the calls was around mean peak frequencies below 32 kHz in both stocks, but in SD rats also another distribution peak occurred around 50 kHz, with call durations typically shorter than 0.3 s and frequency modulations below 5 kHz (**Figures 6B,D**).

Next, we quantified call numbers depending on acoustic call features and divided response calls into those with mean peak frequencies below or above 32 kHz, durations shorter or longer than 0.3 s, and frequency modulations below or above 5 kHz (**Table 1**). This analysis showed that in both stocks the majority of response calls was below 32 kHz. Considering durations, most calls were shorter than 0.3 s, particularly in SD rats. Frequency modulations were mainly below 5 kHz. When comparing the percentages of calls with mean peak frequencies below 32 kHz among stocks, WI rats were found to have higher percentages of calls below 32 kHz ($t_{43} = 2.137$, $p = 0.038$). Considering percentages of calls with durations below 0.3 s, stocks did not differ ($t_{43} = -1.95$, $p = 0.058$). The same was true for frequency modulations. Similar percentages of calls were emitted with modulations below 5 kHz in both stocks ($t_{43} = 0.173$, $p = 0.864$).

In addition, we asked whether response calls below or above 32 kHz were related to each other in individual

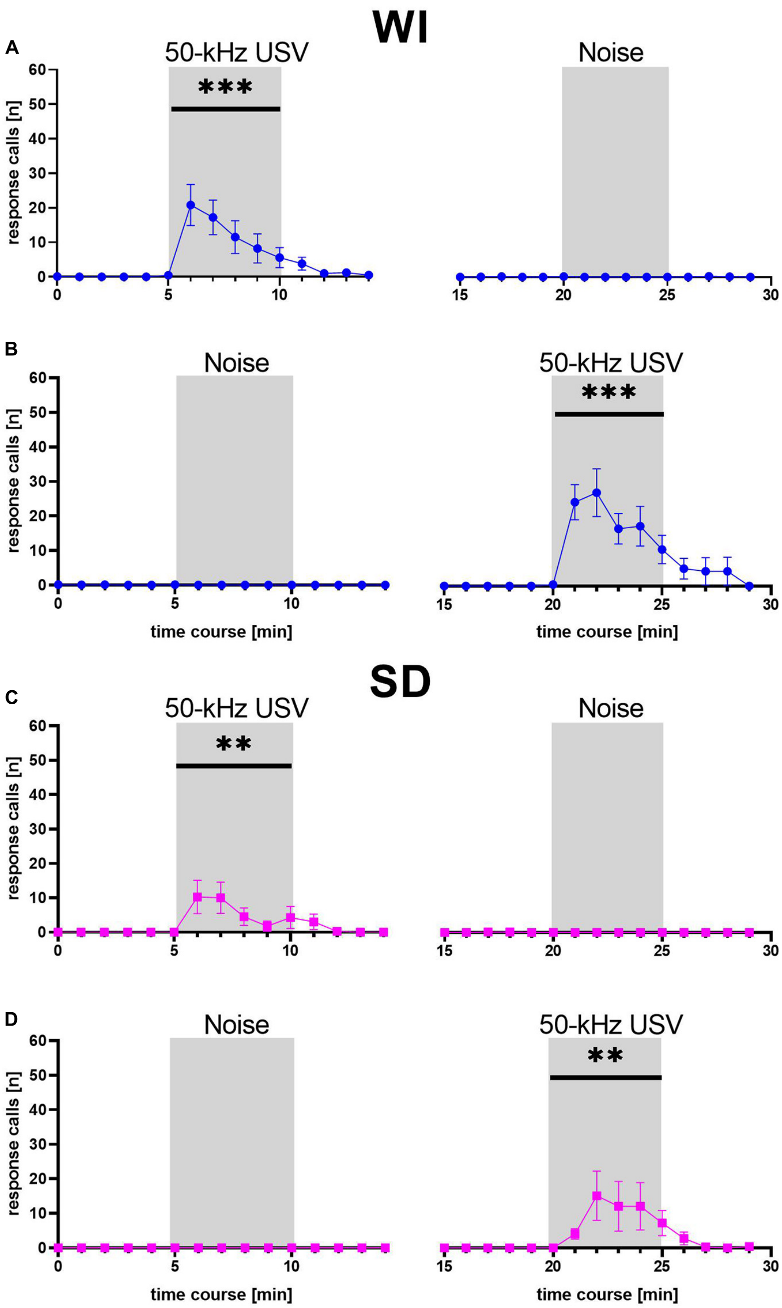

FIGURE 3 | Mean numbers (±SEM) of response calls emitted during each minute of WI (blue dots; **A,B**) and SD (magenta squares; **C,D**) rats. $^{**}p < 0.01$, $^{***}p < 0.001$.

animals (**Figure 7**), but did not find significant correlations between the two in WI ($r = 0.08$, $p = 0.66$) or SD rats ($r = -0.26$, $p = 0.44$).

Relationships Between Response Calls and Playback-Induced Approach

Thirdly, we asked whether the emission of the response calls was correlated with social approach behavior evoked by playback of 50-kHz USV. As stated in the Introduction, the present response call data sets were obtained in a study where social approach behavior evoked by 50-kHz USV playback was examined (Berz et al., 2021). In that study, approach behavior was quantified by subtracting the time spent on the proximal arms (i.e., close to the speaker) before playback from the time spent thereon during the presentation of 50-kHz USV. The same was done for the proximal entries (see detailed analysis in Berz et al., 2021). These numbers were now correlated with the total amount of response calls evoked by playback of 50-kHz USV to see

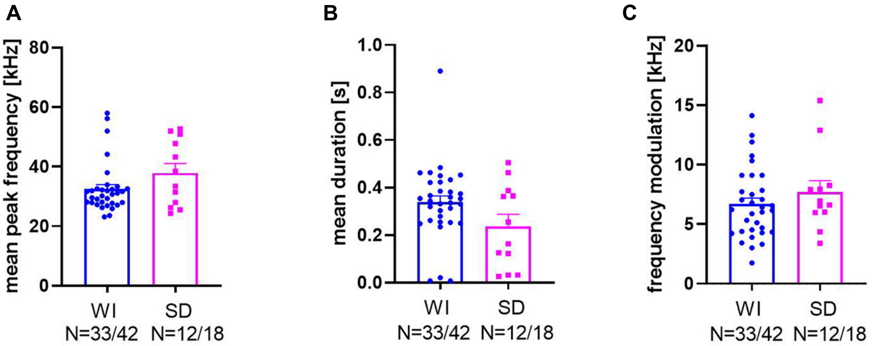

FIGURE 4 | Bar graphs and individual data points of mean peak frequency **(A)**, mean duration **(B)**, and frequency modulation **(C)** of WI (blue dots) and SD rats (magenta squares).

whether social approach behavior was related to the emission of response calls across individual rats. In WI rats, this tended to be the case. The more time the rats spent close to the active speaker, the more calls in response to 50-kHz USV playback they tended to emit ($r = 0.314$, $p = 0.075$). A more prominent correlation was evident in SD rats, where social approach and the emission of response calls were strongly associated (SD: $r = 0.662$, $p = 0.019$). No such correlations were found with respect to proximal arm entries (WI: $r = 0.01$, $p = 0.952$; SD: $r = -0.017$, $p = 0.948$). To test whether these correlations were only a byproduct of locomotor activity during playback, the total numbers of response calls were correlated with the degree of locomotor activation using the distance traveled during playback in comparison to the distance traveled before playback. Neither in WI nor SD rats a correlation was found ($r = 0.065$, $p = 0.681$; $r = 0.151$, $p = 0.551$, respectively). Also, the numbers of response calls were not correlated with locomotor activity during the first 15 min on the maze as a measure of general locomotor activity (WI: $r = 0.031$, $p = 0.864$; SD: $r = 0.187$, $p = 0.540$).

Data Set 3: Effects of Drug Treatment

Call Numbers and Latencies

In the third data set, rats were treated either with the dopaminergic D2 receptor antagonist Halo or saline as a control. The pharmacological treatment had no prominent effect on the emission of response calls and the proportion of vocalizing rats (saline: 15 out of 24, Halo: 20 out of 24) did not differ between Sal and Halo ($x^2{}_{1,\,48} = 2.64$, $p = 0.104$). Moreover, treatment did not affect response call numbers ($t_{46} = 0.465$, $p = 0.644$; **Figure 8A**; Sal: 66.5 ± 31.18; Halo: 86 ± 31.53) and latencies to start calling ($t_{33} = 0.578$, $p = 0.567$; **Figure 8B**; Sal: 19.41 ± 4.18 s; Halo: 26.33 ± 9.86 s).

Temporal Emission Pattern

Similar to the previous data sets 1 and 2, the emission of response calls was strongly dependent on stimulus ($F_{1,\,46} = 11.771$, $p = 0.001$) and stimulus phase ($F_{2,\,92} = 14.443$, $p < 0.001$), with an interaction between stimulus and stimulus phase ($F_{2,\,92} = 14.373$, $p < 0.001$), while treatment had no major impact (treatment: $F_{1,\,46} = 0.194$, $p = 0.662$; treatment × stimulus: $F_{1,\,46} = 0.232$, $p = 0.632$; treatment × stimulus phase: $F_{2,\,92} = 0.842$, $p = 0.434$; treatment × stimulus × stimulus phase: $F_{1,\,92} = 0.797$, $p = 0.454$; **Figure 9**). Specifically, as in the previous data sets 1 and 2, playback of 50-kHz USV but not noise led to a prominent increase in response calls, which occurred during the 5 min of 50-kHz USV playback and up to 5 min thereafter. The peak was again typically seen during the second or third minute after 50-kHz USV playback onset. With onset of 50-kHz USV playback, the numbers of emitted response calls increased significantly in rats treated with Sal ($F_{1,\,23} = 6.443$, $p = 0.018$) but also in rats treated with Halo ($F_{1,\,23} = 8.068$, $p = 0.009$). After that, calling rate decreased to zero at the latest 5 min after playback had ended. Substantial calling only occurred in response to 50-kHz USV and not in response to noise and was therefore specific to the 50-kHz USV playback in both treatment groups (Sal: $F_{1,\,23} = 4.687$, $p = 0.041$; Halo: $F_{1,\,18} = 7.613$, $p = 0.013$). Furthermore, the call emission sequence showed that most animals started calling with higher frequencies around 50 kHz and quickly changed to emit calls of frequencies around 22 kHz (**Supplementary Figure 1B**).

Response Call Features

For a further characterization of response calls in the third data set, their mean peak frequencies, durations, and frequency modulations were analyzed. Sal-treated animals had peak frequencies around 33.76 ± 2.8 kHz, which was not significantly different from Halo-treated animals (30.89 ± 2.49 kHz; $t_{33} = 0.898$, $p = 0.376$; **Figure 10A**). Call durations in controls were 0.282 ± 0.036 s, which was significantly shorter than those of Halo-treated rats (0.395 ± 0.039 s; $t_{33} = 2.048$, $p = 0.049$, **Figure 10B**). Frequency modulation did not differ between treatment groups and Sal-treated rats called with a frequency modulation of 5.33 ± 0.56 kHz compared to 6.16 ± 0.66 kHz in HALO-treated rats ($t_{33} = 0.919$, $p = 0.365$; **Figure 10C**).

The response calls were various in shape and differed in call parameters (for examples of response calls, see **Figure 11**). For better visualization of the different call parameters and the distribution of the individual calls, scatter plots for either call durations or frequency modulations were plotted vs. peak frequencies (**Figure 12**). The accompanying histograms show

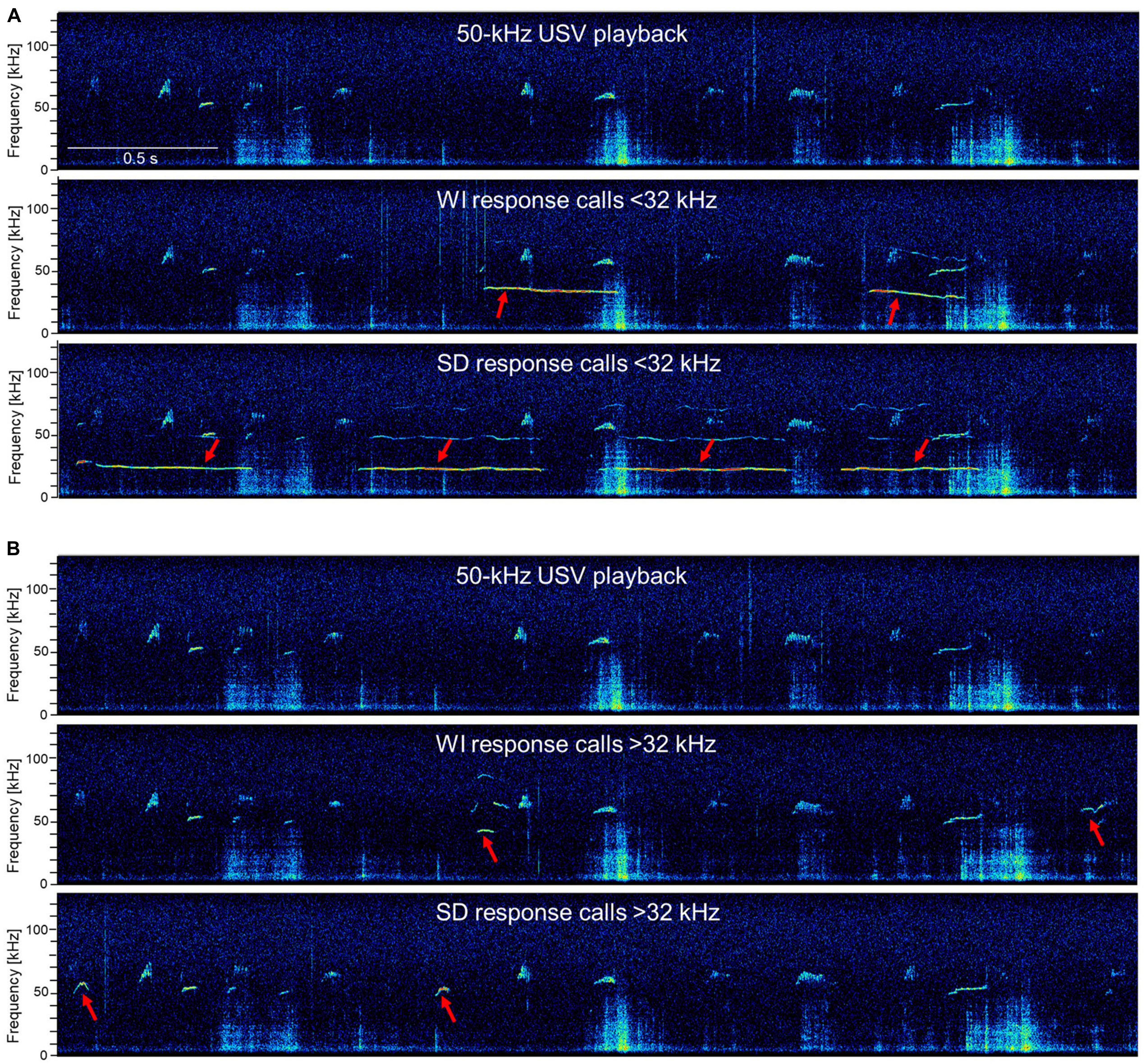

FIGURE 5 | Exemplary response calls during 50-kHz USV playback. The first picture is always the 50-kHz USV playback sequence and the following pictures show response calls in addition to the 50-kHz USV playback sequence (red arrows) < 32 kHz **(A)** or > 32 kHz **(B)** of WI and SD rats. Exemplary high-resolution spectrograms (frequency resolution 488 Hz; time resolution 0.512 ms) were generated with SASLab Pro software 5.2.09 (Avisoft Bioacoustics) *via* fast Fourier transformation (512 FFT length, 100% frame, Hamming window, and 75% time-window overlap).

the main distribution at mean peak frequencies around 25 kHz in both treatment groups; meaning that the majority of calls were below 32 kHz. Especially in Halo-treated rats, few calls were above 32 kHz. Call durations were as well above as below 0.3 s in Sal- and Halo-treated rats. Frequency modulation was mainly below 5 kHz.

Next, we again quantified call numbers depending on acoustic call features and divided response calls into those with mean peak frequencies below or above 32 kHz, durations shorter or longer than 0.3 s, and frequency modulations below or above 5 kHz (**Table 2**). When comparing the percentages of calls with mean peak frequencies below 32 kHz among treatment groups, no significant difference was detected ($t_{33} = -0.978$, $p = 0.335$). Considering durations below 0.3 s, there was likewise no difference ($t_{33} = 1.996$, $p = 0.054$). The same was true for frequency modulations, since similar percentages of calls were emitted with modulations smaller than 5 kHz in both groups ($t_{33} = 0.979$, $p = 0.335$).

In addition, we again asked whether response calls below or above 32 kHz were related in individual animals (**Figure 13**), but found no significant correlations in Sal- ($r = -0.161$, $p = 0.566$) or Halo-treated rats ($r = 0.123$, $p = 0.606$).

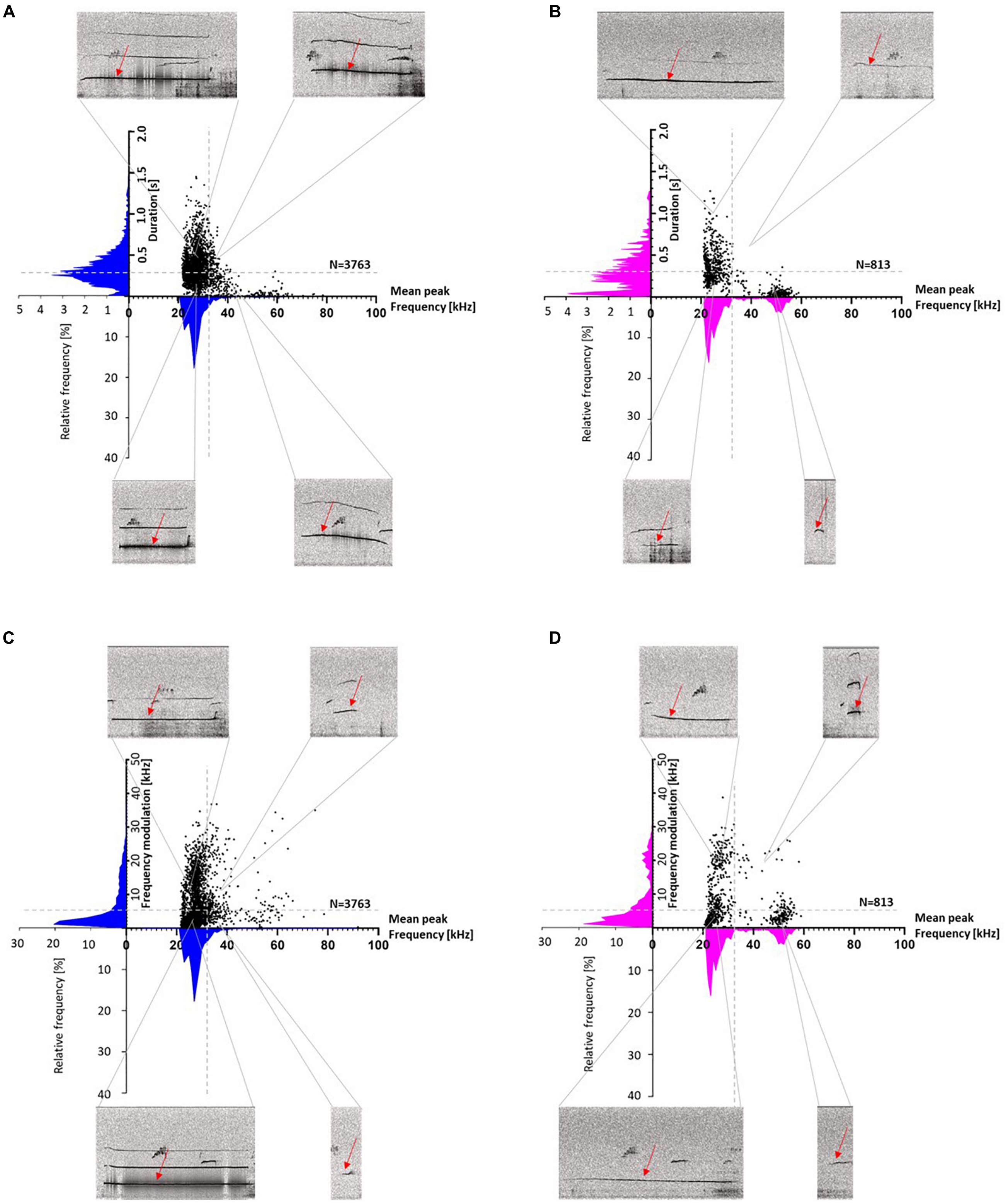

FIGURE 6 | Wistar (WI) **(A,C)** and Sprague-Dawley (SD) rats **(B,D)** scatter plots with histograms (blue for WI and magenta for SD rats) of duration or frequency modulation vs. mean peak frequency. Duration is divided into <> 0.3 s (**A,B**: horizontal gray dashed lines), frequency modulation is divided into <> 5 kHz (**C,D**: horizontal gray dashed lines) and mean peak frequencies are divided into <> 32 kHz (vertical gray dashed line). For each section, an exemplary call with the respective parameters is shown (red arrows).

TABLE 1 | Scatter plot distributions for Wistar (WI) and Sprague-Dawley (SD) rats.

WI ***N* = 33/42**			**Mean peak frequency**		
			=32 kHz	**> 32 kHz**	**Total calls**
	Total numbers (percentages) means ± SEM		3,328 (88.44%) 69.34 ± 5.61	435 (11.56%) 30.66 ± 5.61	3,763 (100%)
Duration	<0.3 s	1,936 (51.44%) 48.69 ± 4.5	1,644 (43.7%)	292 (7.8%)	
	>0.3 s	1,827 (48.56%) 51.34 ± 4.5	1,684 (44.8%)	143 (3.8%)	
Modulation	<5 kHz	2,272 (60.38%) 51.62 ± 4.18	2,104 (55.9%)	168 (4.5%)	
	>5 kHz	1,491 (39.62%) 48.38 ± 4.18	1,224 (32.5%)	267 (7.1%)	
SD ***N* = 12/18**			**Mean peak frequency**		
			=32 kHz	**>32 kHz**	**Total calls**
			599 (73.7%) 44.29 ± 11.95	214 (26.3%) 55.71 ± 11.95	813 (100%)
Duration	<0.3 s	479 (58.9%) 66.79 ± 9.2	273 (33.6%)	206 (25.3%)	
	>0.3 s	334 (41.1%) 33.21 ± 9.2	326 (40.1%)	8 (1%)	
Modulation	<5 kHz	464 (57.1%) 50.21 ± 7.62	324 (39.9%)	140 (17.2%)	
	>5 kHz	349 (42.9%) 49.79 ± 7.62	275 (33.8%)	74 (9.1%)	

Mean peak frequencies < or > 32 kHz, Durations = or > 0.3 s, frequency modulations = or > 5 kHz.

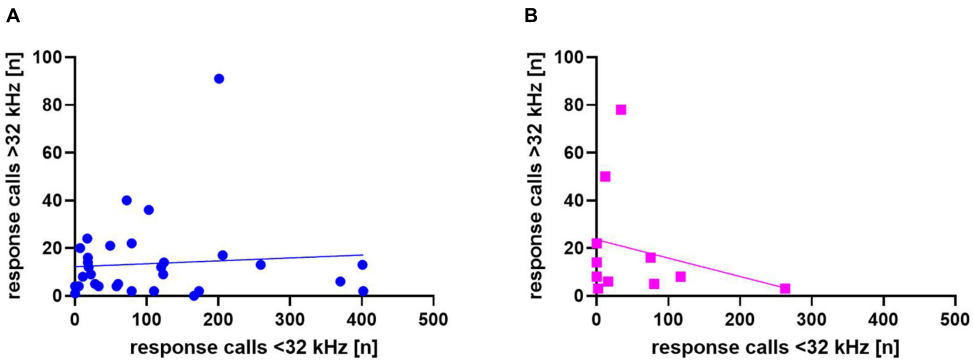

FIGURE 7 | Correlation between calls <32 kHz and >32 kHz for Wistar (WI) **(A)** and Sprague-Dawley (SD) **(B)** rats. Each data point represents response calls below and above 32 kHz of one animal.

Relationships Between Response Calls and Playback-Induced Approach

To see whether social approach was associated with the emission of response calls, these two parameters were again correlated. The results were the same in both treatment conditions. In Sal-treated rats, there were no significant correlations, neither between the time spent in the proximal arms close to the active speaker nor between the entries into those with the amount of response calls (Sal time: $r = -0.0195$, $p = 0.487$; Sal entries: $r = 0.059$, $p = 0.783$). In Halo-treated animals, likewise no significant correlations between proximal time or entries and number of emitted calls were detected (Halo time: $r = 0.143$, $p = 0.547$; Halo entries: $r = -0.112$, $p = 0.602$). Moreover, locomotor activity during 50-kHz USV playback in comparison to the distance traveled before playback was not correlated with the total numbers of response calls, irrespective of treatment condition (Sal: $r = -0.101$, $p = 0.639$; Halo: $r = -0.113$, $p = 0.598$). In addition, locomotor activity during the first 15 min on the platform was not correlated with the number of response calls (Sal: $r = -0.224$, $p = 0.421$; Halo: $r = 0.238$, $p = 0.312$).

DISCUSSION

In this study, we characterized response calls emitted by rats exposed to playback of appetitive 50-kHz USV, previously shown to function as social contact calls (Wöhr, 2018). The phenomenon that rats respond to playback of species-specific 50-kHz calls by emitting response calls has been repeatedly reported before, but has not been described in detail yet (Wöhr and Schwarting, 2007, 2009; Willadsen et al., 2014; Willuhn et al., 2014; Engelhardt et al., 2017, 2018; Berg et al., 2018, 2021; Kisko et al., 2020; Olszyński et al., 2020, 2021; for an overview see **Supplementary Table 1**). First, we described the emission of response calls in reaction toward 50-kHz USV playback in

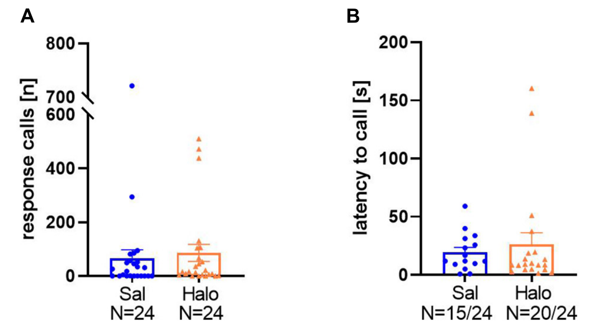

FIGURE 8 | Total numbers of response calls **(A)** and latencies to call **(B)** in Sal- and Halo-treated rats. Data are presented as individual results and as means ± SEM.

WI rats. Secondly, we compared these to SD rats. Thirdly, we analyzed the effect of blocking DA receptors on response calls using Halo, as compared to vehicle-injected WI subjects.

Through these means, we could demonstrate that most rats emitted response calls. Importantly, the emission of response calls was clearly linked to 50-kHz USV playback. In fact, response calls were seen specifically in response to 50-kHz USV but not in response to time- and amplitude-matched noise, replicating previous results (Willadsen et al., 2014; Willuhn et al., 2014; Engelhardt et al., 2017, 2018; Berg et al., 2018, 2021; Kisko et al., 2020; Olszyński et al., 2020, 2021). When exposed to 50-kHz USV, receiver rats often started emitting response calls within the first minute of playback and emission rates were typically peaking after around 2–3 min, often outlasting playback for up to 5 min. This certainly supports naming these calls “response calls.”

Most response calls were characterized by peak frequencies below 32 kHz, the threshold typically applied to differentiate between 22- and 50-kHz USV (Brudzynski, 2001). Although peak frequencies were highly variable and ranged roughly between 20 and 80 kHz, the vast majority of response calls occurred in a frequency range of 20–32 kHz. Similarly, call durations were characterized by large variability, ranging from a few milliseconds to up to 1.5 s. Call durations of about 0.3 s occurred at a particularly high rate. Frequency modulations were typically below 5 kHz. When comparing these values to the parameters of typical 22- and 50-kHz USV, our values correspond more to 22-kHz USV; more precisely the short 22-kHz USV type since the durations were rarely longer than 0.3 s (Brudzynski, 2021).

The emission of response calls was seen in WI and SD rats, suggesting that this is a robust phenomenon not dependent on stocks. Specifically, we found that there were no substantial differences between WI and SD rats, concerning numbers of emitted calls, latencies to start calling, and call likelihood. In both stocks there was a large variability among response calls. However, their mean peak frequencies, call durations, and frequency modulations did not differ significantly between experimental conditions. SD rats only differed in one aspect by clearly showing calls around frequencies of 50 kHz, which was not that prominent in WI rats. This is somehow in line with other studies that also showed higher emission of 50-kHz USV and elevated rough-and-tumble play behavior in SD compared to WI rats (Manduca et al., 2014). Other studies, however, found that WI rats emitted more 50-kHz USV compared to SD rats (Schwarting, 2018a,b), indicating that WI rats are more prone to emit USV in general, which is also not represented by our data. If at all, on a descriptive level, WI rats emit slightly less response calls compared to SD rats. Regarding call parameters, previous studies showed marginal differences between stocks, i.e., shorter call durations in SD rats compared to WI rats (Schwarting, 2018b). On a descriptive level again, this aligns with our results, albeit this difference in call duration did not yield significance. Apart from stock differences, various other factors like breeding or experience have to be taken into account. Moreover, inter-individual differences should not be neglected, as our results also suggest (Schwarting, 2018a,b).

In our study, the pharmacological treatment with the D2 antagonist Halo did not affect call likelihood, call rates, latencies, temporal distribution, peak frequency, and frequency modulation. In Sal-treated WI rats, the majority of calls was again below 32 kHz, however, in Halo-treated rats this was even more prominent and Halo treatment also led to longer call durations. Previous studies showed that exposure to 50-kHz USV playback under the influence of systemically applied amphetamine, a catecholaminergic agonist, resulted in response calls with frequencies around 50 kHz at the expense of 22 kHz (Engelhardt et al., 2017). Specifically, calls of lower frequencies decreased drastically under the influence of amphetamine. In contrast, response calls in the 50 kHz range increased dose-dependently following amphetamine administration. This is in line with a large number of studies showing that the emission of 22- and 50-kHz USV are associated with the activation of distinct neurotransmitter systems (for review: Brudzynski, 2021). While 22-kHz USV are associated with the cholinergic system (Brudzynski, 2001; Kroes et al., 2007; Willadsen et al., 2018), the dopaminergic system plays an important role in the regulation of 50-kHz USV (Wöhr, 2021). For instance, electrolytic or 6-hydroxydopamine lesions of the ventral tegmental area reduce the emission of 50-kHz USV (Burgdorf et al., 2007). Conversely, emission of 50-kHz USV can be evoked by electrical stimulation of the ventral tegmental area or the nucleus accumbens (Burgdorf et al., 2000, 2007). Moreover, psychostimulants, most notably amphetamine, lead to a robust increase in 50-kHz USV emission (Rippberger et al., 2015). Additionally, playback of 50-kHz USV was shown to induce enhanced levels of activity in the nucleus accumbens (Sadananda et al., 2008), where it elicits a rapid phasic release of dopamine (Willuhn et al., 2014). Based on these findings, one could have assumed that the dopaminergic receptor blockade with Halo should decrease response call numbers, especially those above 32 kHz, which was apparently not the case. Possibly, these calls are not critically dependent on dopamine D2 receptor function, and might be dependent on endogenous opiates, as indicated by an earlier playback study with the opiate receptor antagonist naloxone (Wöhr and Schwarting, 2009).

Together, the present findings indicate that the emission of response calls is a robust phenomenon that is seen specifically in response to playback of 50-kHz USV independent of stock and despite blocking dopamine neurotransmission. These

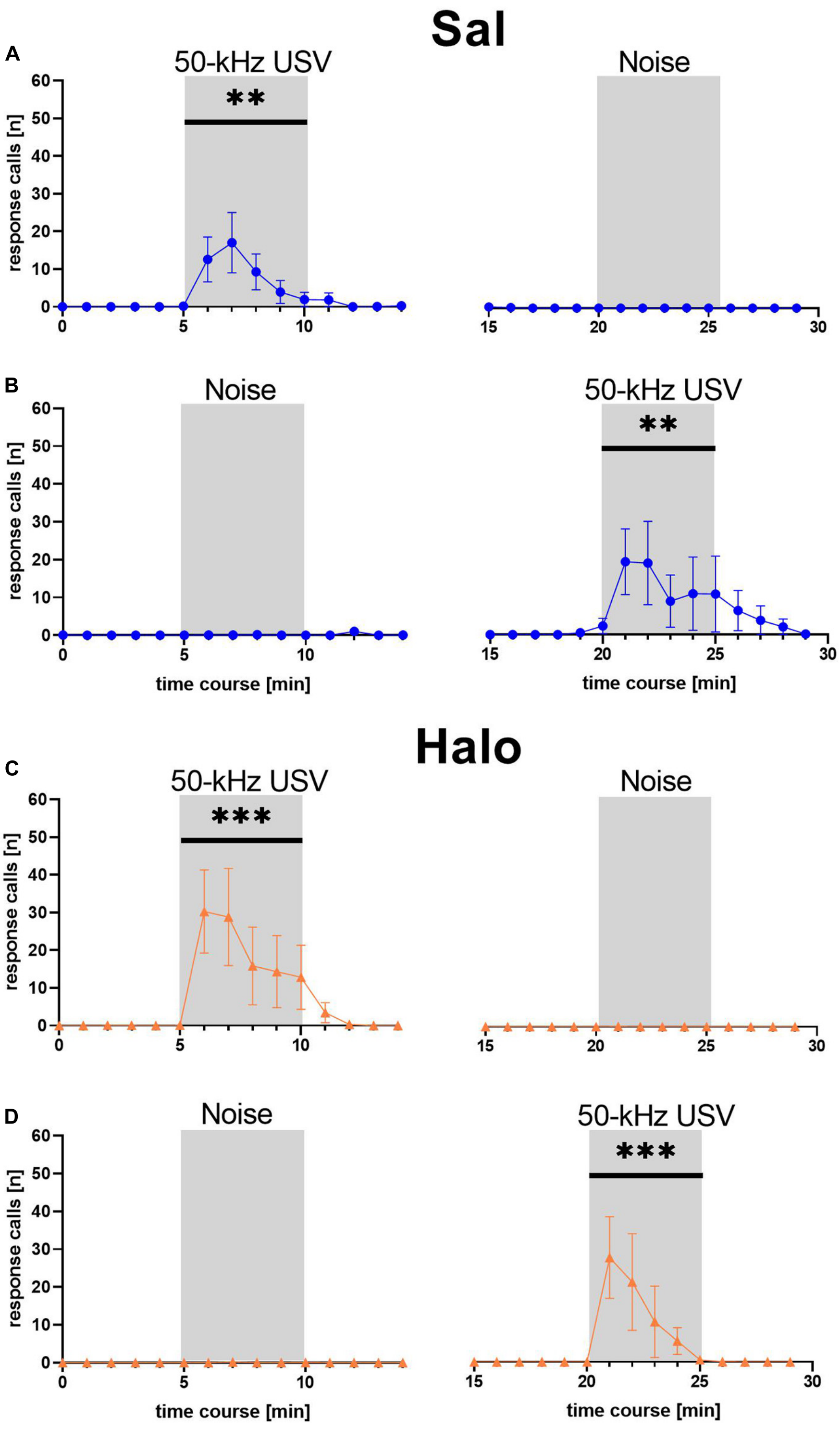

FIGURE 9 | Mean number of response calls emitted during each minute of Sal- (blue; **A,B**) and Halo-treated (orange; **C,D**) rats. Most calls were emitted during 50-kHz USV stimulus and almost no calls were emitted during noise. $^{**}p < 0.01$, $^{***}p < 0.001$.

observations are in line with the idea that the emission of response calls reflects changes in affect that are caused by playback of 50-kHz USV. For example, one might expect the induction of a positive affective state in response to appetitive 50-kHz USV. On the other hand, it was suggested that response calls reflect frustration induced by the inability to reach the

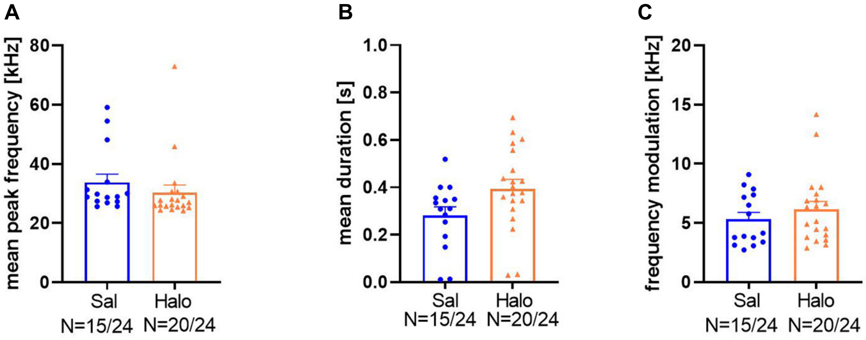

FIGURE 10 | Call parameters of Sal- (blue) and Halo-treated (orange) rats for mean peak frequency **(A)**, mean duration **(B)**, and frequency modulation **(C)**.

conspecific emitting 50-kHz USV. Alternatively, response calls might serve communicative functions as social contact calls or as appeasement signals. While the present findings do not allow drawing strong conclusions about causes and functional significance of response calls, they provide first insights into potential mechanisms underlying their emission.

In support of the idea that response calls might reflect an affective state we hypothesize that the rats are not solely in one affective state, but rather in an ambivalent state. There is convincing evidence in support of the notion that USV emission reflects prominent affective states (Brudzynski, 2021) and that different call types are associated with distinct states (Brudzynski, 2013b). Because USV below 32 kHz are typically believed to function as alarm or distress calls reflecting a negative affective state, this would suggest that playback of 50-kHz USV induced a negative state in the receiver rats. However, the strong level of social approach behavior and the emission of 50-kHz response calls, at least in SD rats, evoked by playback of 50-kHz USV speaks against the induction of a solely negative affective state through 50-kHz USV playback (Wöhr, 2018). Furthermore, the positive and negative emotional states in rats were proposed to be mutually exclusive and acting in an antagonistic manner (Brudzynski, 2021). It is possible, however, that the two states quickly alternate which leads to the hypothesis of an ambivalent state, with negative and positive phases present in an oscillating manner. This is also reminiscent of an approach/avoidance conflict, i.e., a situation characterized by choices leading to either reward or punishment (Aupperle et al., 2015). Interestingly, it was shown that rats emit 22-kHz as well as 50-kHz USV during neutral situations and not only aversive ones (Robakiewicz et al., 2019). The study by Robakiewicz et al. (2019) also showed that both call types and hence presumably both emotional states can be present during an emotional neutral task of performing nose pokes in order to change the light of the experimental apparatus. Both call types were also found in a cocaine self-administration task (Barker et al., 2010), where animals received either high or low doses of cocaine. Low dose rats predominantly emitted short 22-kHz calls and high dose rats emitted mostly 50-kHz calls. Nevertheless, both groups showed calls of both emotional states and this supports the hypothesis of the ambivalent state. In the present study, however, only SD rats emitted 50-kHz USV to a higher extent and all other experimental groups mainly emitted calls with frequencies below 32 kHz. Additionally, the emissions of response calls below and above 32 kHz were not correlated across individual rats, suggesting that there was no general tendency for emitting response calls in both frequency ranges, which speaks against the hypothesis of an ambivalent state.

With respect to the emission of 22-kHz calls, this phenomenon might be explained by the hypothesis of a frustrated state in the receiver rat, possibly induced by the violated expectation of another rat being present. Other studies suggested that short 22-kHz calls (<0.3 s) represent a dysphoric state or displeasure without any external threat (Simmons et al., 2018), which is in line with the mean peak frequencies, durations, and low frequency modulations of the response calls found in our study. This might also be an indication that calls with low frequencies in response toward 50-kHz USV playback are an expression of internal distress, i.e., frustration, as suggested before (Wöhr and Schwarting, 2009). Frustration is defined as a result of behavior after an expected but not received reward (Scull et al., 1970; Burokas et al., 2012). In our playback paradigm, the rat probably realized that there was no rat physically present for interaction after hearing the 50-kHz USV playback, and this could have led to a state of frustration in the approaching rat. This might also explain why the majority of response calls was emitted within 2 or 3 min after the onset of the 50-kHz USV playback. At first, the animals heard and recognized the stimulus, exhibited a strong social approach immediately afterward and as soon as the rats realized that there was no conspecific present, the emission of response calls increased as an expression of a frustrated state. In line with the frustrated state hypothesis is our finding that the first calls of most animals of data set 2 and 3 were of higher frequencies, i.e., around 50 kHz and quickly changed to calls with frequencies in the 22-kHz USV range (**Supplementary Figure 1**).

On the other hand, the positive correlation of response calls and approach behavior might serve the hypothesis that the response calls could also be characterized as social contact calls. 50-kHz USV have been postulated to fulfill an affiliative communication function to, for example, maintain a playful state during rough-and-tumble play or as social contact calls to reestablish social proximity after separation of conspecifics (Wöhr et al., 2016). An indication that the response calls in

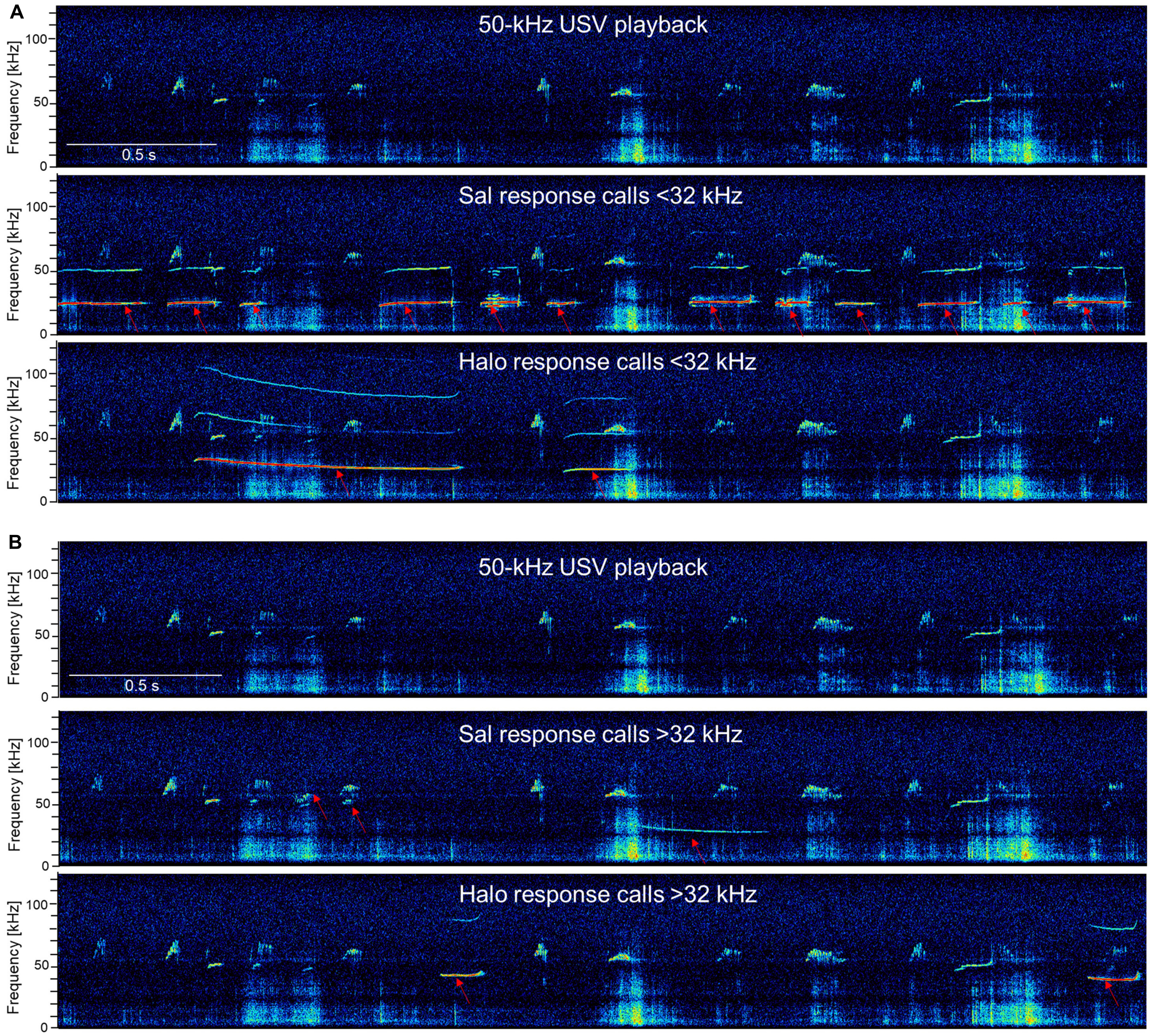

FIGURE 11 | Exemplary response calls during 50-kHz USV playback. The first picture is always the 50-kHz USV playback sequence and the following pictures show response calls in addition to the 50-kHz USV playback sequence (red arrows) <32 kHz **(A)** or >32 kHz **(B)** of Sal- and Halo-treated rats. Note that the calls depicted for Sal- or Halo-treated rats are not specific to the treatment groups and calls were descriptively similar in all groups.

our study serve as social contact calls is that they are emitted during social approach behavior. Further, such calls are emitted frequently during the approach behavior like 50-kHz USV during rough-and-tumble play (Knutson et al., 1998). In our study we found a moderate positive correlation between response calls and approach behavior, i.e., the time spent close to the active speaker, in SD and, at least to some extent, in WI rats. Apparently, the more the animals tried to reach a possible conspecific signaled by the 50-kHz USV playback, the more calls they emitted, supporting the hypothesis of response calls being contact calls. For Sal- and Halo-treated WI rats, however, this was not the case. In Halo-treated rats, the absence of a positive correlation between approach behavior and response call emission was probably due to the drug-induced immobility (Berz et al., 2021). Since Sal-treated rats also received an i.p. injection 60 min prior to testing, this might have influenced their approach response, as well as their calling behavior; even though Sal-treated rats significantly approached the sound source (Berz et al., 2021) and emitted similar numbers of response calls as WI rats. No correlation was found, however, between overall activity and call numbers in any group. Also or alternatively, the positive correlation between approach behavior and response calls especially observed in SD rats might not be in order to establish contact, but rather due to hypervigilance. Olszyński et al. (2021) showed that in response to 50-kHz USV playback, heart rate and locomotor activity increased as well as the emission of USV. The USV in response

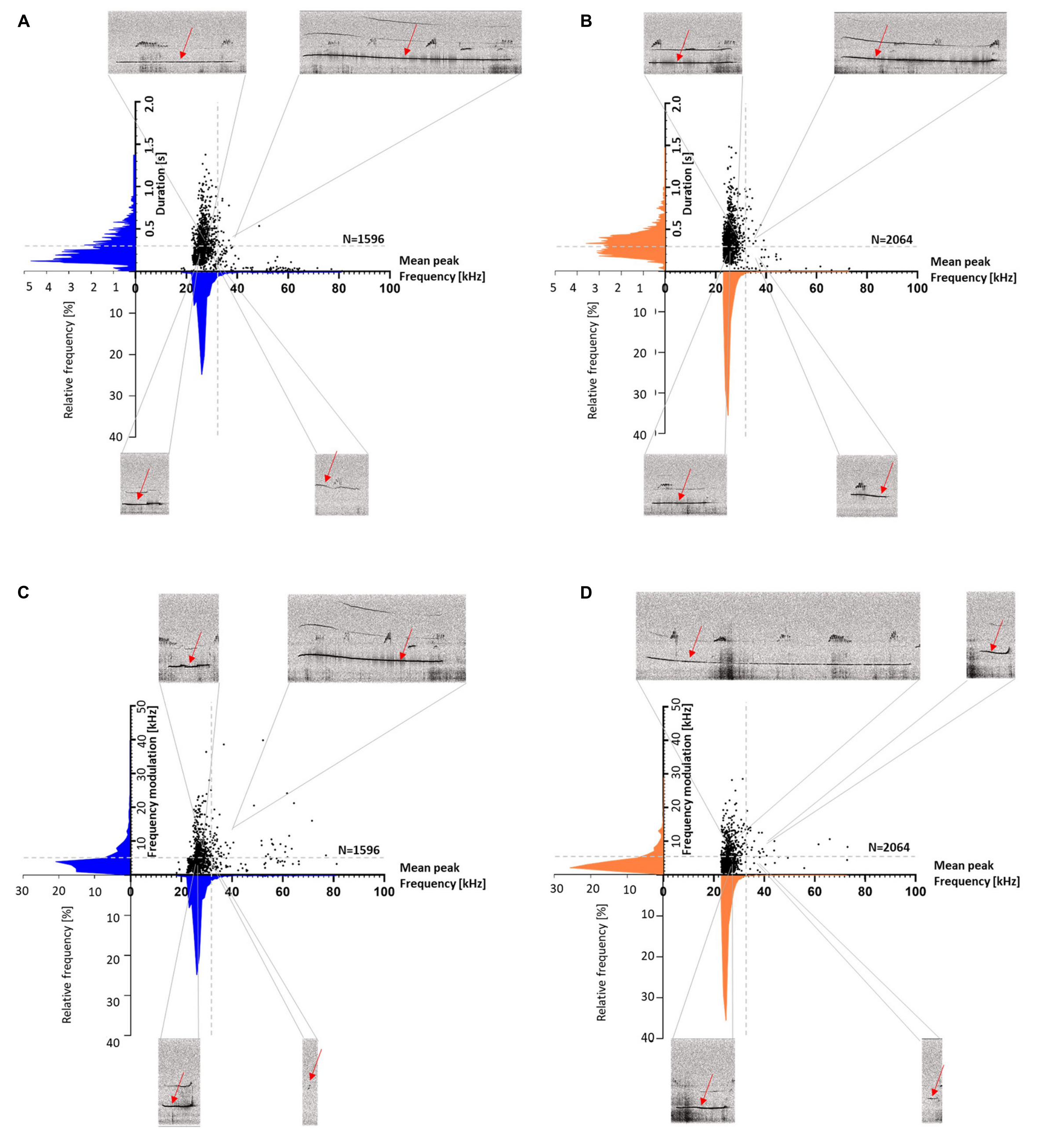

FIGURE 12 | Sal- **(A,C)** and Halo-treated rats **(B,D)** scatter plots with histograms (blue for Sal- and orange for Halo-treated rats) of duration or frequency modulation vs. mean peak frequency. Duration is divided into <> 0.3 s (**A,B**: horizontal gray dashed lines), frequency modulation is divided into <> 5 kHz (**C,D**: horizontal gray dashed lines) and mean peak frequencies are divided into <> 32 kHz (vertical gray dashed line). For each section, an exemplary call with the regarding parameters is shown (red arrows).

to 50-kHz USV playback in that study were mainly 50-kHz calls, possibly representing contact calls, in contrast to our study here, where the animals mostly emitted calls of lower frequencies. Also, the peak of call emission occurred shortly after the recipient of the playback was in proximity to the sound source and ceased after playback has stopped, which suggests that these calls could function to establish social contact or in search of it. However, the response calls linked to the 50-kHz USV playback do not classify as 50-kHz calls because their mean peak frequencies are much lower, the duration is longer, and there is hardly any frequency modulation compared to 50-kHz calls.

Alternatively, response calls could serve appeasing purposes. The age difference between the rat of the recorded playback and the test subject might be of interest, because in our study, a juvenile rat heard 50-kHz USV playback recorded from an adult rat and accordingly, it seems plausible for the

TABLE 2 | Scatter plot distributions for Sal- and Halo-treated rats.

Sal ***N* = 15/24**			**Mean peak frequency**		
			=32 kHz	**>32 kHz**	**Total calls**
	Total numbers (percentages) means ± SEM		1,490 (93.4%) 72.77 ± 8.78	106 (6.6%) 27.23 ± 8.78	1,596 (100%)
Duration	<0.3 s	1,044 (65.4%) 59.39 ± 5.91	960 (60.2%)	84 (5.3%)	
	>0.3 s	552 (34.6%) 40.61 ± 5.91	530 (33.2%)	22 (1.4%)	
Modulation	<5 kHz	1,147 (71.9%) 60.27 ± 6.73	1,098 (68.8%)	49 (3.1%)	
	>5 kHz	449 (28.1%) 39.73 ± 6.73	392 (24.6%)	57 (3.6%)	
Halo ***N* = 20/24**			**Mean peak frequency**		
			=32 kHz	**> 32 kHz**	**Total calls**
			2,021 (97.9%) 83.44 ± 6.78	43 (2.1%) 16.56 ± 6.78	2,064 (100%)
Duration	<0.3 s	919 (44.5%) 40.48 ± 6.89	893 (43.3%)	26 (1.3%)	
	>0.3 s	1,145 (55.5%) 59.52 ± 6.89	1,128 (54.7%)	17 (0.8%)	
Modulation	<5 kHz	1,633 (79.1%) 50.76 ± 6.73	1,624 (78.7%)	9 (0.4%)	
	>5 kHz	431 (20.9%) 49.24 ± 6.73	397 (19.2%)	34 (1.6%)	

Mean peak frequencies < or > 32 kHz, Durations = or > 0.3 s, frequency modulations = or > 5 kHz.

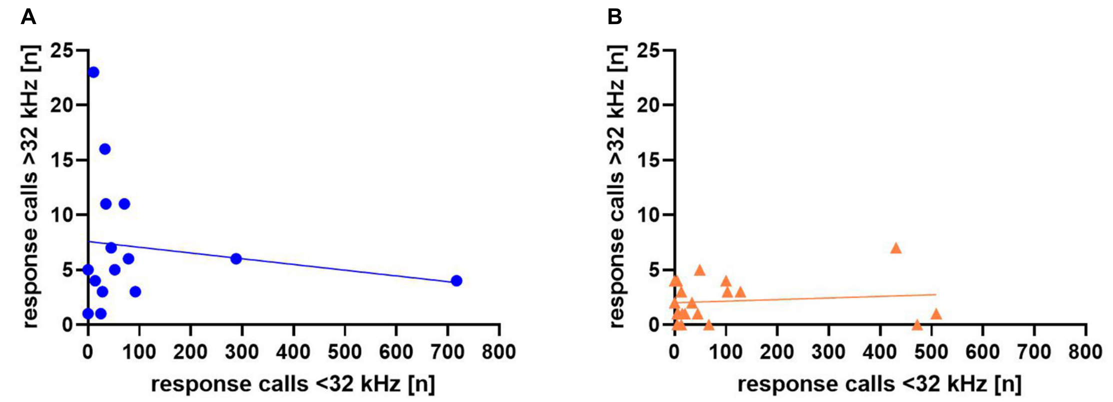

FIGURE 13 | Correlation between calls <32 kHz and >32 kHz for Sal- **(A)** and Halo-treated **(B)** rats. Each data point represents response calls below and above 32 kHz of one animal.

subject rat to cautiously approach the potential conspecific. Supporting this hypothesis, is the fact that in adult male rats, USV calls of lower frequencies were found during play fighting (Burke et al., 2017, 2020). In social situations that were at risk to escalate into aggression, the play partners lowered their calls gradually from 50 kHz to around 30 kHz with increasing durations (Burke et al., 2017). The authors hypothesized that this group of calls might be a transition from 50-kHz flats to 22-kHz flats or a unique new type of calls. The function of these calls is probably the induction of appeasement, i.e., to de-escalate a situation at risk to turn into aggression (see also Sales, 1972; Lore et al., 1976). Our results seem to support this hypothesis since we tested juvenile rats subjected to calls from an older adult rat and the response calls were in similar frequencies. Moreover, the response calls had also similar frequency modulations, like the calls in the study by Burke et al. (2017) and were not exclusively flat as the common 22-kHz USV. So far, however, it is not known whether receiver rats can gain information about the age of the sender based on their USV.

Importantly, the response call phenomenon studied here in detail appears sufficiently robust to be used as a measure for the reciprocal nature of acoustic communication and can easily be applied in rat model systems for neuropsychiatric disorders, where acoustic communication is impaired, such as autism spectrum disorder (Lai and Baron-Cohen, 2015). In preclinical studies examining USV with the aim to reveal communication deficits in rodent model systems, most laboratories have focused exclusively on the sender. Although there is now an increasing number of preclinical studies including playback paradigms to learn about the responses evoked in the receiver as well (Berg et al., 2018, 2020a,b; Kisko et al., 2018, 2020; Wöhr et al., 2020), an important aspect of acoustic communication that is often still neglected is its reciprocal nature and the fact that a signal emitted by the sender frequently evokes the emission of a response signal in the receiver (Seyfarth and Cheney, 2003). Measuring response calls offers a unique opportunity to overcome this limitation. It offers a new approach to studying the reciprocal nature of communication in rodent models for neuropsychiatric disorders.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Tierschutzbehoerde, Regierungspraesidium Giessen, Germany, TVA No. 35-2018.

AUTHOR CONTRIBUTIONS

RS and MW designed the study, acquired resources and funding, and oversaw the project. AB performed the experiments. AB with substantial help from MW analyzed the data. AB, RS, and MW wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2021.812142/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling editor declared a past co-authorship with one of the authors MW.

Appendix

Table S1: Chi^2 test results (X^2) for animals emitting response calls [#] between first test and retest of all experimental groups. A p-value <0.05 was considered significant (*).

Experimental group (caller/ non-caller)	First test [#]	Retest [#]	X^2
AMPH-treatment before the retest			
AMPH caller	9	4	X^2=5.495 p=0.019*
AMPH non-caller	1	6	
SAL caller	10	5	X^2=6.667 p=0.0098*
SAL non-caller	0	5	
Stock comparison			
WI caller	12	5	X^2=5.461 p=0.019*
WI non-caller	6	13	
SD caller	10	8	X^2=0.444 p=0.505
SD non-caller	8	10	
State-dependency with HALO treatment			
HALO-HALO caller	8	2	X^2=6.171 p=0.013*
HALO-HALO non-caller	4	10	
SAL-SAL caller	7	1	X^2=6.75 p=0.009*
SAL-SAL non-caller	5	11	
HALO-SAL caller	10	3	X^2=8.224 p=0.004*
HALO-SAL non-caller	2	9	
SAL-HALO caller	7	2	X^2=4.444 p=0.035*
SAL-HALO non-caller	5	10	

Gene and protein nomenclature

SHANK3	Gene symbol in humans
Shank3	Gene symbol in rodents
SHANK3	Protein symbol in humans and rodents
UBE3A	Gene symbol in humans
Ube3a	Gene symbol in rodents
UBE3A	Protein symbol in humans and rodents
CACNA1C	Gene symbol in humans
Cacna1c	Gene symbol in rodents
CACNA1C	Protein symbol in humans and rodents

Abbreviations

5-HTT	Serotonin transporter
AMPH	d-amphetamine
AS	Angelman Syndrome
ASD	Autism spectrum disorder
BLA	Basolateral amygdala
D2	Dopamine receptor D2
DA	Dopamine
HALO	Haloperidol
IGF-II	Insuline-like growth factor II
kHz	Kilohertz
min	Minutes
ms	Milliseconds
NAc	Nucleus accumbens
PND	Postnatal day
s	seconds
SAL	Saline
SD	Sprague-Dawley rats
SNc	Substantia nigra pars compacta
USV	Ultrasonic vocalizations
ViolEx 2.0	ViolEx model 2.0
VTA	Ventral tegmental area
WI	Wistar rats

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Erklärungen

Eigene Beiträge der Veröffentlichungen

Entsprechend §9 (1) der Promotionsordnung der Philipps-Universität Marburg (Fassung mit Ausführungsbestimmungen des Promotionsausschusses des FB Psychologie vom 31.01.2020) werden im Folgenden die eigenen Anteile an den einzelnen Publikationen erläutert.

Study I: Limited generalizability, pharmacological modulation, and state-dependency of habituation towards pro-social 50-kHz calls in rats.

Berz A, Pasquini de Souza C, Wöhr M, Schwarting RKW (2021). Limited generalizability, pharmacological modulation, and state-dependency of habituation towards pro-social 50-kHz calls in rats. *iScience 24, 102426.*

- Verteilung der prozentualen Anteile:
    - Annuska Berz: 70 %
    - Camila Pasquini de Souza: 5 %
    - Markus Wöhr: 10 %
    - Rainer Schwarting: 15 %

Study II: Response calls evoked by playback of natural 50-kHz ultrasonic vocalization in rats.

Berz AC, Wöhr M, Schwarting RKW (2022). Response calls evoked by playback of natural 50-kHz ultrasonic vocalization in rats. *Frontiers in Behavioral Neuroscience 15:812142*.

- Verteilung der prozentualen Anteile:
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Unterzeichnung des ersten Gutachters und der Verfasserin:

Prof. Dr. Rainer K.W. Schwarting

Annuska C. Berz

Eidesstattliche Erklärung

Hiermit versichere ich, dass ich die vorliegende Dissertation:

„Playback of ultrasonic vocalizations in rats: habituation, response calls, and drug effects"

Selbstständig, ohne unerlaubte Hilfe angefertigt habe und mich keiner anderen als der von mir ausdrücklich bezeichneten Quellen und Hilfen bedient habe.

Die Dissertation wurde in der jetzigen oder einer ähnlichen Form noch bei keiner anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

Marburg, ______________________________

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Annuska C. Berz