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

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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
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; Olzyński 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...
to be more pronounced in juveniles 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; 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; Schönfeld et 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...
access to the relevant memory trace behave similar to rats lacking such a memory trace due to treatment with an amnesic drug (Wohr 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
particularly prominent in the initially more active Wistar rats, while Sprague-Dawley rats showed a moderate decline in locomotor activity (stock x 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 ($t_{17} = 3.387$, $p = .001$).
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 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\textsubscript{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\textsubscript{1,68} = 18.917, p < .001 and F\textsubscript{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\textsubscript{17} = 2.389, p = .029 and t\textsubscript{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\textsubscript{17} = 0.413, p = .685 and t\textsubscript{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\textsubscript{17} = 2.706, p = .011 and t\textsubscript{17} = 2.400, p = .022, respectively), while distal arm entries and the time spent on distal arms did not differ (t\textsubscript{17} = 0.995, p = .327 and t\textsubscript{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\textsubscript{17} = 1.782, p = .093 and t\textsubscript{17} = 1.015, p = .324; respectively) but led to decreased proximal arm entries and the time spent on proximal arms were less pronounced during the second playback in Wistar rats (t\textsubscript{17} = 0.413, p = .685 and t\textsubscript{17} = 0.313, p = .758; respectively). Together, this indicates that Wistar rats do not show a social approach response 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).

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\textsubscript{19} = 1.905, p = .036; times spent: t\textsubscript{19} = 10.360, p < .001), together with decreases in distal arm behavior (arm entries: t\textsubscript{19} = 2.164, p = .022; times spent: t\textsubscript{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\textsubscript{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\textsubscript{1,18} = 29.871, p < .001; main effect time: F\textsubscript{14,252} = 15.507, p < .001; time x drug interaction: F\textsubscript{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 ( \pm SEM): call duration 40 \pm 0.8 ms, peak frequency 62.986 \pm 4.219 kHz, and frequency modulation 23.666 \pm 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 \pm SEM: 7065 \pm 737 vs. 6110 \pm 544 cm), but these subgroups did not differ significantly from each other (t\textsubscript{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.
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 (t9 = 0.737, p = .480), with a trend for the time spent on proximal arms (t9 = 2.023, p = .074). Also, there were no significant changes to noise playback (all p values >.05; except for time spent on distal arms: t9 = 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 (t9 = 3.244, p = .010) and distal entries (t9 = 2.299, p = .047), but tended to be more pronounced in case of proximal arm entries as compared to distal ones (t9 = 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 (t9 = 2.370, p = .042), while the time spent on distal arms was not affected (t9 = 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 (t9 = 3.971, p = .003), while proximal arm entries did not differ (t9 = 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 (t18 = 3.119, p = .006), albeit this was also the case for distal arm entries (t18 = 2.158, p = .045) but not the time spent on proximal and distal arms (t18 = 0.753, p = .461 and t18 = 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: F14,616 = 15.704, p < .001), which was more prominent in the SAL-treated groups (time × drug interaction: F14,616 = 5.057, p < .001), most likely because HALO-treated groups displayed very low levels of locomotor activity from the beginning (main effect drug: F3,44 = 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: F14,616 = 16.979, p < .001), again primarily in the SAL groups, together with drug treatment effects (main effect drug: F3,44 = 166.246, p < .001; time × drug interaction: F42,616 = 4.062, p < .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: F1,22 = 5.667, p = .026; time × drug interaction: F14,308 = 1.600, p = .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: F1,22 = 6.711, p = .017; time × drug interaction: F14,308 = 8.284, p < .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 (t23 = 2.534, p = .019), together with a higher dwell time in the proximal zone (t23 = 6.288, p < .001) and a lower dwell time in the distal zone (t23 = 2.178, p = .040). In response to noise, proximal and distal zone entries were reduced (t23 = 2.524, p = .019 and t23 = 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).
Centered dwell time in the distal zone (t = 2.523, p = .028). Less dwell time in the distal zone entries: t = 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 so-

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 = 3.563, p = .004) and spent more time there in proximity to the active ultrasonic speaker during 50-kHz USV playback (t = 9.006, p < .001), together with less dwell time in the central zone (t = 2.523, p = .028). Less dwell time in the distal zone was also evident during noise playback (t = 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 50-kHz USV playback (t = 2.409, p = .035), the time spent in proximity to the active ultrasonic speaker was not significantly higher than before playback (t = 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 = 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 = 2.409, p = .035), the time spent in proximity to the active ultrasonic speaker was not significantly higher than before playback (t = 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 = 1.534, p = .152; zone entries: t = 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 exper-

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%; chisq = 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 (Wörh 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: Wörh 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 (Wörh 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 (Wörh 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 (Schoenfeld 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 (Woehr 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; Wohr 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 (Wohr 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 Wohr (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 Wohr 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

Annuska Berz, Camila Pasquini de Souza, Markus Wöhr, and Rainer K.W. Schwarting
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).

Radial maze playback paradigm (Experiments 1 and 2): 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).

Platform playback paradigm (Experiment 3): 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
habitation 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.
Supplementary References

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