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Lesions of the rat basolateral amygdala reduce the behavioral response to ultrasonic vocalizations

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ABSTRACT

Rats emit vocalizations in the ultrasonic range (ultrasonic vocalizations; USVs), of which 50-kHz USVs could communicate positive affective states and induce approach behavior in conspecifics, whereas 22-kHz USVs might signal negative affective states and potential threats. Listening to 50-kHz USVs can be rewarding, but it is unknown which brain mechanisms are responsible for the assignment of reinforcing value to 50-kHz USVs . The behavioral responses induced by listening to 22-kHz USVs are heterogeneous and need further characterization. The amygdala is a region relevant for social perception, behavior and reward. Here, we tested the hypothesis that the basolateral amygdala (BLA) plays a causal role in motivating behavioral responses to 50-kHz and 22-kHz USVs. Rats with lesions of the BLA or sham lesions were repeatedly exposed to playback of either 50-kHz or 22-kHz USVs in a radial maze. Compared to sham rats, BLA-lesioned rats spent less time in the arms close to the USV speaker during playback of both 50-kHz or 22-kHz USVs. This difference in behavior was not due to impaired motor or general auditory abilities, indicating that BLA lesions selectively reduced the responsiveness to stimuli with social significance. This finding provides further support for the hypothesis that the BLA plays an important role in motivating approach behavior to social reinforcers.

1. Introduction

Rodents emit calls in the ultrasonic range to express affect and transmit situation-specific information. In the rat, these ultrasonic vocalizations (USVs) can be categorized into three main classes: 50-kHz USVs are emitted during social play [1,2], after administration of amphetamine [3]and mating or tickling [4], thus in appetitive states. By contrast, 22-kHz USVs are emitted in more aversive situations, such as depression-like states [5], exposure to a predator [6] and fear learning [7]. The third type, 40-kHz USVs, are emitted by pups during social isolation [8].

There is vast consensus that USVs have a social-communicative function and affect the behavior of conspecifics. For instance, 22-kHz USVs emitted in threatening situations might serve as alarm calls to alert conspecifics [9]. Likewise, 50-kHz USVs are emitted in situations of close social contact, such as rough-and-tumble-play and mating, but also seem to function as motivators to engage in such behaviors [10,11].

Interestingly, playback of previously recorded USVs, even in the absence of another rat, can also influence behavior. Using an eight-arm radial maze, Wöhr and Schwarting [12] showed that both juvenile and adult rats transiently increased locomotor activity and spent more time close to the speaker during and after the playback of 50-kHz USVs. Importantly, social exploratory activity and approach behavior was not seen in response to non-USV background noise and other acoustic control stimuli, concluding that 50-kHz USVs induced transient behavioral activation and approach behavior [2,12–14]. By contrast, 22-kHz USV playback caused weak, but significant behavioral inhibition [12,15–17]. In another experiment, it was shown that rats deliberately self-administered playback of 50-kHz, but not 22-kHz USVs [4]. In line with those findings are recent results from our group where rats preferred a compartment associated with 50-kHz USV playback to a compartment associated with background noise playback [18]. In sum, this body of research suggests that USVs are socially salient stimuli. While 22-kHz USVs might carry informational value about potential threats, listening to 50-kHz USVs might be rewarding for rats. This

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hypothesis is supported by a recent study showing that playback of prerecorded 50-kHz, but not 22-kHz USVs or background noise, elicits increased neural activity [19] and dopamine release [20] in the rat nucleus accumbens, one of the core regions in the reward circuitry of the brain.

However, it is not completely understood which brain regions support the assignment of incentive value to USVs. It is unlikely that the nucleus accumbens is the only site where motivational significance is attributed to social signals, since it is a general motivational output region responsive to both social and non-social reinforcement. Another candidate area is the amygdala. Rat amygdala neurons represent 50-kHz and 22-kHz USVs [21–23] and the amygdala is known to play a central role in the processing of rewards, emotions and decision making (for review see [24]). For example, the different nuclei of the amygdala have been implicated in fear conditioning [25–27] and additionally a crucial role of the amygdala in reward representation and reinforcement learning has been suggested [28,29]. Recently, the role of the amygdala in social cognition came into focus. In humans, the amygdala was found to be important for social perception, social reward and social behavior in general (for a detailed review, see [30]). Amygdala lesions in neonatal rats severely disturbed social behavior during adulthood [31,32], whereas the same lesions induced in adult rats only impaired specific types of social learning [33]. In addition, single neuron activity in the primate basolateral amygdala (BLA) encodes rewards to self and others [34]. Finally, research from our group has shown that lesions of the BLA negatively impacted prosocial behavior in rats [35]. While control rats preferred mutual-reward outcomes, *i.e.* sucrose pellets delivered both to themselves and a conspecific, to own-reward outcomes, *i.e.* no reward to the conspecific, rats with BLA lesions were indifferent between mutual- and own-reward outcomes. Interestingly, BLA-lesioned rats did not show abnormalities when discriminating between different reward magnitudes, indicating that the deficit induced by the lesions was selective to social decision making [35]. This finding suggests that BLA-lesioned rats failed to attach positive value to rewards delivered to partners. One tentative explanation is that BLA-lesioned rats became indifferent to the motivational aspect of social signals, such as USVs, which normally drive prosocial behavior in non-lesioned rats [36].

The aim of the present experiment was to further clarify the role of the BLA in motivating behavioral responses to USVs in rats. More specifically, we tested the hypothesis that BLA lesions selectively reduce the typical orientation response and the transient approach behavior towards 50-kHz USVs. To this end, we compared behavioral changes in response to playback of 50-kHz and 22-kHz USV stimuli of BLA-lesioned rats with the behavior of sham-lesioned rats.

2. Materials & methods

2.1. Subjects

The experiment was conducted according to the European Union Directive 2010/63/EU for animal experiments and approved by the local authority (Landesamt für Natur, Umwelt und Verbraucherschutz North-Rhine Westphalia, Germany). Thirty-two male Long-Evans rats (Charles River, Italy), about ten weeks old and weighting 379 ± 5 g at the date of surgery, were housed in pairs under a reversed 12 h day/night cycle. The housing room was kept at a constant temperature of 22 °C and a humidity of 60%. Throughout the experiment, rats received standard laboratory rodent food (Sniff, Germany) and water *ad libitum*.

2.2. Surgical procedures

Rats from each cage were pseudorandomly assigned to receive either a lesion of the BLA or sham surgery, resulting in n = 16 rats with a BLA lesion and n = 16 rats without a comparable lesion. Prior to surgery, rats received analgesia (5 mg/kg carprofen subcutaneously) and anesthesia was induced with 5% isoflurane, followed by 1.5–5% isoflurane to maintain anesthesia. Once under anesthesia, rats were placed in a stereotactic frame (David Kopf Instruments, USA) and received a local anesthetic at the incision side and behind the ears (0.5 mg bupivacaine in total, subcutaneously). To apply bilateral excitotoxic BLA or sham lesions, two small holes were drilled bilaterally into the skull at the following coordinates: anterior-posterior (AP) 2.4 mm posterior to bregma, medial-lateral (ML) ± 4.8 mm lateral to midline (site 1) and AP 3.0 mm posterior to bregma and ML ± 4.8 mm lateral to midline (site 2 [35]). Microinfusions were done at dorsal-ventral (DV) 8.6 mm ventral to dura (site 1) and DV 8.8 mm ventral to dura (site 2) for both the left and right BLA. At each injection side, 0.36 mm wide needles (PlasticsOne, USA), connected to a 10 µl Hamilton syringe fixed in a microinfusion pump (Harvard Apparatus, USA), were lowered to the target depth in succession. Rats in the BLA lesion group received injections of 0.2 µl of 0.09 M quinolinic acid in 0.1 M phosphate buffer (pH 7.2) at a speed of 1 µl/min per site. Upon completion of the injection, the needle was kept in place for 2 additional minutes to ensure complete diffusion of the substance before needle retraction. For animals in the sham group, the injection procedure was identical to that of the BLA lesion group, except that sham animals received injections of 0.2 µl 0.1 M phosphate buffer (pH 7.2) at all four injection sides. After completion of the injections, the incision was sutured and animals were left to recover for at least one week including post-operative analgesia on two successive days (carprofen 5 mg/kg).

2.3. Behavioral testing

2.3.1. Setup

We used an eight-arm radial maze similar to a maze previously employed [12]. The radial maze consisted of a central platform (36 cm diameter) and eight arms (14 cm wide and 60 cm long) that spread out from the central platform in a star-shaped pattern. For behavioral analyses the *center* was designated to consist of the central platform and the entry area of each arm (approximately one third of the whole length of the arm; shown in light grey in Fig. 1). In order to play USV stimuli, a speaker (customized Ultrasonic Speaker Vifa, Avisoft) was positioned 20 cm away from the end of one arm (active speaker). To prevent any bias caused by the mere presence of the speaker (*i.e.* the animals might spend some time exploring the speaker), a second speaker was placed at the end of the opposite arm (inactive speaker). The three arms closest to the active speaker were denoted as the *proximal arms*, the three arms closest to the inactive speaker were denoted as the *distal arms* and the two remaining arms were denoted as *neutral* (Fig. 1). The sizes of the areas of interest relative to the total surface of the maze are as follows: center 37.3%, proximal and distal arms each 23.5% and neutral arms 15.7%. It has to be noted that the chance to stay in the center, even during active exploratory movement, might be somewhat higher due to its larger surface area. In addition, rats had to pass through the center in order to transition from one arm to another, thereby further increasing the total amount of time spent in the center.

During a testing session, only one of the two speakers emitted acoustic stimuli (played by using Bioacoustics Recorder version 4, Avisoft). In addition, the speaker-arm assignment was pseudorandomized within each test session to ensure that each of the arms was designated as an active arm during a single session. Testing sessions were recorded with a camera centered above the maze to allow offline analysis of the behavior.

2.3.2. Acoustic stimuli

Three types of acoustic stimuli were presented to each rat for one minute: Stimuli containing 50-kHz USVs, 22-kHz USVs and background noise, as described elsewhere [12]. Shortly, 50-kHz USV stimuli consisted of a 3.5 s sequence containing 13 natural 50-kHz USVs, which were repeated for a total duration of one minute. Twenty-two-kHz USV stimuli consisted of a sequence of 29 natural USVs that also lasted one

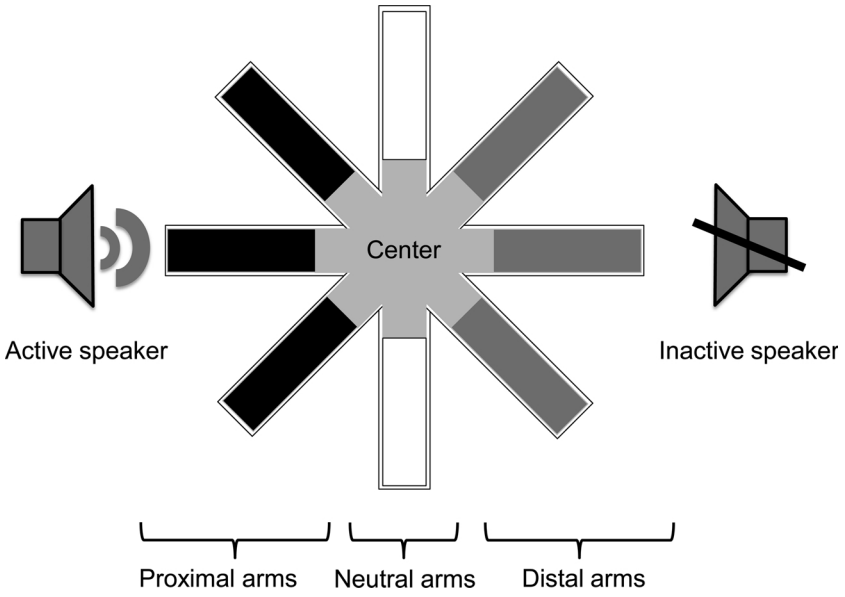

Fig. 1. Schematic drawing of the radial maze.
The radial maze consists of a central platform (center; light grey) and eight arms spreading out from the platform. The three arms close to the active speaker (emitting background noise and USV playback) are designated as "proximal arms" (black), the three arms furthest from the active speaker are the "distal arms" (dark grey) and two remaining arms with a neutral orientation ("neutral arms"). In the arm opposite to the one with the active speaker, a second speaker was placed (inactive speaker), which did not emit any acoustic stimuli and served to control for non-specific effects of the presence of a speaker.

minute. Note that the natural 22-kHz USVs had a longer call duration compared to the natural 50-kHz USVs used in the sequences (average call duration 1.18 s for 22-kHz USVs and 0.07 s for 50-kHz USVs). Unlike the individual USVs within the 50-kHz USV stimulus, none of the individual 22-kHz USVs was repeated within the respective stimulus. It is assumed that 22-kHz USVs are emitted in a specific order of precedence and repeating individual calls within a sequence would disturb their informational content [12]. Finally, background noise contained sounds of a rat walking over cage bedding material without emitting any USVs.

2.3.3. Task design

Behavioral testing started approximately ten days after surgery. We adopted the task design from Wöhr and Schwarting [12] with minor modifications, to measure approach, avoidance or immobilization responses to acoustic stimuli. At the start of the session and before each playback phase (see below for the description of task phases), the rats were (re-)positioned in the middle of the central platform with their head facing the inactive speaker. We recorded the locomotor behavior of rats in response to 50-kHz or 22-kHz USV playback emitted from a speaker at the end of a randomly selected arm in the radial maze. Playback of 50- or 22-kHz USVs was repeated during three consecutive test sessions for each rat. Each test session consisted of five phases: (1) habituation to the maze during 15 min without any playback (*habituation*), (2) playback of background noise during 1 min (*background playback*), (3) a 10-minute inter-stimulus-interval (ISI) without any acoustic stimuli (*background ISI*), (4) playback of either 50-kHz or 22-kHz USVs during 1 min (*USV playback*) and lastly (5) a 10-min ISI without any USVs present (USV ISI).

All rats were exposed to both 50-kHz and 22-kHz USV playback on separate testing days. Half of all rats from each group started with the exposure to 50-kHz USV playback, whereas the other half started with the exposure to 22-kHz USV playback. The order of task conditions was determined pseudorandomly. Exposure to each type of USV stimulus was repeated on three consecutive test sessions, one session a day. For example, a rat starting in the 50-kHz USV playback condition was exposed to 50-kHz USVs once a day in sessions 1–3, followed by exposure to 22-kHz USVs in sessions 4–6. A rat starting in the 22-kHz condition was first exposed to 22-kHz USVs in sessions 1–3, followed by 50-kHz USVs in sessions 4–6.

2.3.4. Behavioral analysis

Each test session was video-recorded and each recorded file of the respective session was cut into 5 fragments corresponding to the 5 phases in one session. Using tracking software (Ethovision XT version 11.5, Noldus), the movement of the rats on the maze was digitized and the following parameters were assessed, as done by Wöhr and Schwarting [12]: the *total duration* of each rat spent in a specific set of arms and the center and the *transition frequency* from the central platform into the arms. Both parameters were calculated for the proximal arms and for the distal arms (Fig. 1). The time spent in the proximal arms during USV presentation was interpreted as approach behavior, the time spent in the distal arms as avoidance behavior. Both parameters were computed separately and transformed into standardized values relative to the duration of the respective phase. The raw total duration was used to calculate the percentage total duration for each phase, by dividing each raw value by the duration of the respective phase and multiplying it by 100. The raw transition frequencies were standardized through division by the duration of each respective phase. To assess potential motor abnormalities induced by the BLA lesions, the general motor parameter *total distance moved* was assessed for exploration of the entire maze.

2.4. Statistical analyses

All statistical analyses were carried out using SPSS Statistics (version 24; IBM, USA). Data were analyzed using several mixed analyses of variance (ANOVAs) with USV playback (50-kHz *vs*. 22-kHz), USV stimulus repetition sessions (sessions 1–3), arms (proximal *vs*. distal) or testing phase (background playback *vs*. background ISI) as within-subjects factors, group (lesion *vs*. sham) as between-subjects factor on the percentage total duration spent in the proximal and distal arms, the transition frequencies into the respective arms and total distance moved as dependent variables. Bonferroni correction was applied to all post hoc analyses.

2.5. Histology

Rats were transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer and brains were stored in the fixation solution until further processing. Coronal sections were cut at a thickness of 45 µm using a vibratome (Leica) and stained with 1% cresyl violet perchlorate in order to visualize the location of the lesion. Lesions were identified as an accumulation of apoptotic cells within the BLA, as evaluated by a blind experimenter, similar to a previous study by our group [35]. In short, the blind experimenter identified the presence of

lesions within the BLA and surrounding areas, their location and judged whether this respective rat belonged to the BLA lesion or sham group. Exclusion criteria were unilateral, incomplete and/or misplaced lesions in the BLA lesion group and lesions in the sham group comparable to lesions observed in the BLA group.

3. Results

3.1. Bilateral injections of quinolinic acid caused local apoptosis in the BLA

In the BLA lesion group, a greater amount of apoptotic cells was observed bilaterally in the BLA compared to the sham group. All lesion animals had lesions that involved substantial portions of the BLA and occasionally damage to adjacent amygdalar nuclei, such as the lateral and central amygdala. The blind experimenter could successfully assign the animals to their respective groups, except four animals within the sham group. These animals showed lesions in the BLA that might have resulted from track marks made during the injection procedure and, after closer inspection, appeared different from the apoptotic lesions observed in the BLA lesion group. Data were analyzed with and without these sham animals with potential BLA damage, yielding comparable results. Therefore, we did not exclude the respective animals from the behavioral analyses. Pictures of a representative excitotoxic lesion and a sham lesion are shown in Fig. 2A and B. To further define the location and specificity of the excitotoxic lesions, we delineated all areas with apoptotic cells or gross tissue damage in brain slices close to the injection sides and displayed a sum of all lesions at the target area in Fig. 2C. Areas in light grey illustrate the lesions of individual animals, areas in dark grey show regions where lesions of three or more animals overlap. Some cortical and striatal damage due to the injection procedure was detected in animals from both the sham and the lesion groups; however, rats in the lesion group had additional damage to the BLA and surrounding amygdalar nuclei, most prominent in the anterior portion, which was absent in the sham group.

3.2. All rats decreased their approach behavior after repeated exposure to 50-kHz USV stimuli

All rats showed robust approach behavior to the source of 50-kHz, but not 22-kHz USV playback (see below for details). To assess whether the behavior of the rats towards the USV stimuli stayed stable across the three sessions, we performed a mixed ANOVA on the total duration spent in the proximal and distal arms separately during the USV playback phase, comparable to previous analyses [12]. USV stimulus type (50- *vs.* 22-kHz) and test session (sessions 1–3) served as within-subjects factors and lesion (BLA lesion *vs.* sham) as between-subjects factor. For the proximal arms, we found a significant interaction effect of USV type with session on the time spent in those arms during the USV playback phase [$F(2,58) = 14.39$; $p < .001$]. Post hoc analyses with Bonferroni correction showed that rats significantly reduced their time spent in the proximal arms during 50-kHz USV playback across test sessions [$F(2,58) = 24.70$; $p < .001$; Fig. 3A], but not during 22-kHz USV playback (Fig. 3B). During 50-kHz USV playback, rats significantly decreased their time in the proximal arms between the first and the second or third session ($p < .001$ for both session 1 *vs.* session 2 and session 1 *vs.* session 3; Fig. 3A). Likewise, for the distal arms, we found significant main effects of session [$F(2,58) = 6.62$; $p < .01$] and USV type [$F(1,29) = 6.46$; $p < .05$] on the time spent in those arms during USV playback. Post hoc analyses revealed that rats spent a significantly increasing amount of time in the distal arms between sessions 1–3 ($p < .01$; Fig. 3C and D) and the total duration spent in the distal arms was higher during the playback of 22-kHz USVs ($p < .05$). This altered response to USVs after repeated exposure is common and typically reported in the literature [20,37]. Because of this habituation effect, we will only analyze behavior during the first test session.

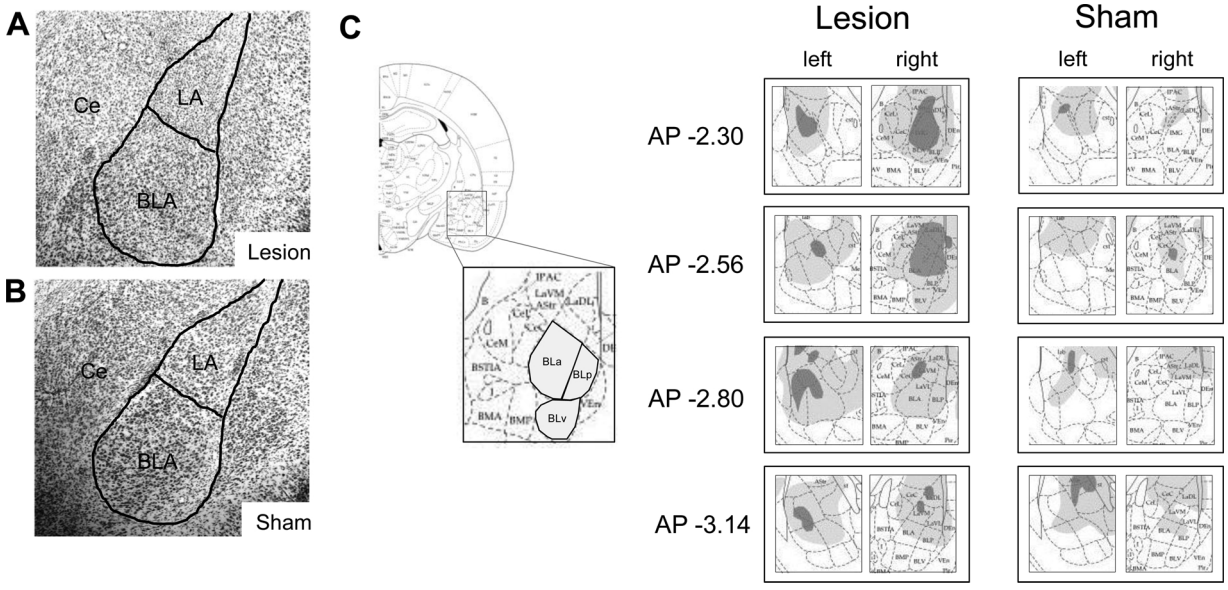

Fig. 2. Injections of quinolinic acid into the BLA caused an increase in the number of apoptotic cells, most pronounced in the anterior BLA.
A and B: Representative micrographs of the BLA in an animal belonging to the lesion group and an animal belonging to the sham group. Both pictures are taken approximately at AP 2.50 mm posterior to bregma. Note the decrease in cell size and cell number in the lesion animal as a result of the quinolinic acid injections. C: Magnified selection showing relevant parts of the BLA at AP 2.56 mm posterior to bregma. The same selection was used for schematic representations of the cumulative lesion spread in the left and right hemisphere of animals belonging to the lesion or sham group. Tissue damage was shown at four bregma levels that are in proximity to the two injection spots (AP 2.40 mm and 3.00 mm posterior to bregma). Light grey areas show the additive tissue damage in all animals, dark grey areas show regions where tissue damage of three or more animals overlapped. In the sham group, some tissue damage is evident at the dorsal border of the amygdala due to track marks of the injection needle, whereas damage to the BLA and surrounding amygdalar nuclei is specific to the lesion group and largest in the anterior BLA. AP: anterior-posterior, BLA: basolateral amygdala, LA: lateral amygdala, Ce: central amygdala, BLa: anterior basolateral amygdala, BLp: posterior basolateral amygdala, BLv: ventral basolateral amygdala; adapted from Paxinos & Watson (1997).

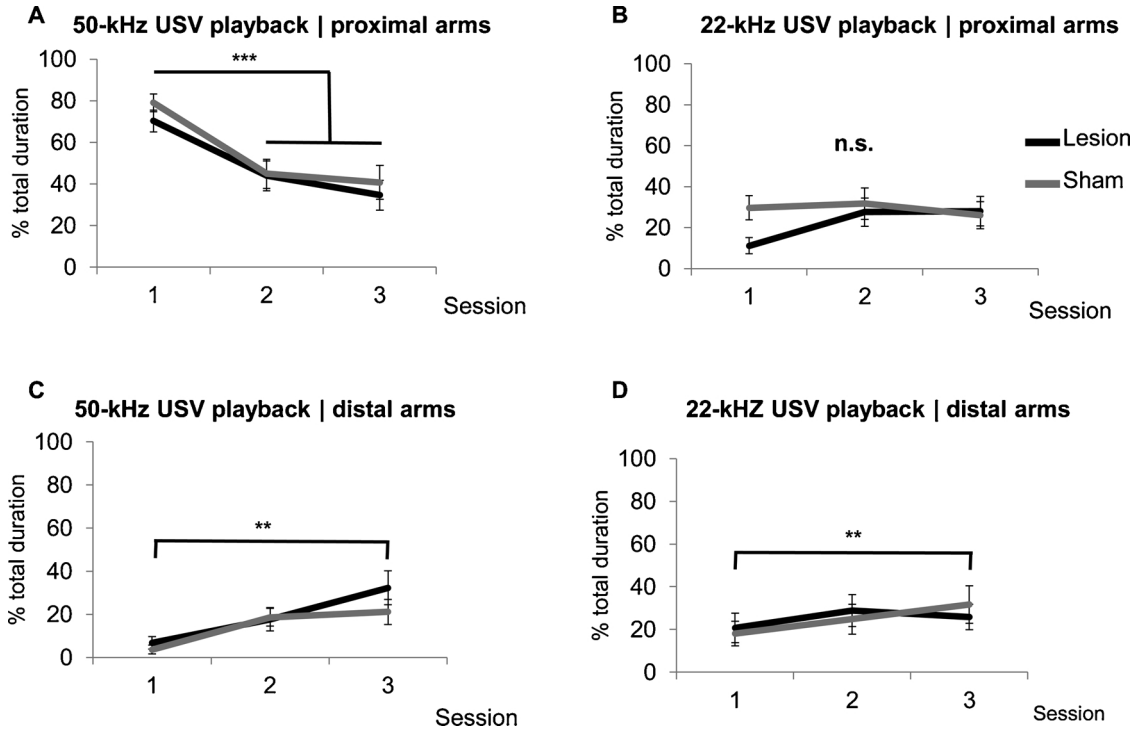

Fig. 3. Repeated exposure to 50-kHz USV playback decreased the total duration of time spent in the proximal arms.
A: Rats significantly decreased the time spent in the proximal arms after initial exposure to 50-kHz USV playback. B: A significant change in behavior was not observed during repeated 22-kHz playback. C and D: Concerning the distal arms, rats increased the total duration of time spent in the arms across sessions. In addition, the total duration spent in the inactive arms was higher during the playback of 22-kHz USVs compared to playback of 50-kHz USVs ($p < .05$). Data are shown as mean ± SEM. ***$p < .001$, **$p < .01$, n.s.: not significant.

3.3. BLA lesions caused an attenuated behavioral response to USV stimuli during the first playback exposure

To measure approach responses to USV playback, we assessed the total duration of time spent in the proximal and distal arms separately, during the first test session. A mixed ANOVA with USV type (50- *vs*. 22-kHz USVs) as within-subjects factor and lesion (BLA lesion *vs*. sham) as between-subjects factor revealed significant main effects of USV type [$F(1,29) = 100.47$; $p < .001$] and lesion [$F(1,29) = 10.82$; $p < .01$] on the total duration spent in the proximal arms during USV playback. All rats spent significantly more time in the proximal arms during playback of 50-kHz USVs compared to playback of 22-kHz USVs during the first test session. In addition, BLA-lesioned rats spent overall less time in the proximal arms compared to sham rats during playback of both 50- and 22-kHz USVs (Fig. 4A). For the distal arms, we found a significant main effect of USV type on total duration [$F(1,29) = 8.46$; $p < .01$], indicating that rats spent less time in the distal arms during 50-kHz than during 22-kHz USV playback during the first test session (Fig. 4B). However, there were no significant differences between BLA-lesioned and sham rats in the total duration of time spent in the distal arms during USV playback [$F(1,29) = 0.36$; $p > .05$, Fig. 4B]. In addition, during the first USV playback, all rats showed a significantly higher frequency of transitions from the center of the maze into the proximal arms when 50-kHz USVs were played compared to playback of 22-kHz USVs [$F(1,29) = 22.16$; $p < .001$; Fig. 5A] whereas a significant effect was not detected for the distal arms [$F(1,29) = 1.48$; $p > .05$; Fig. 5B]. The transition frequencies did not differ significantly between BLA lesion and sham rats during the first test session [$F(1,29) = 1.04$; $p > .05$ and $F(1,29) = 0.29$; $p > .05$; Fig. 5A and B].

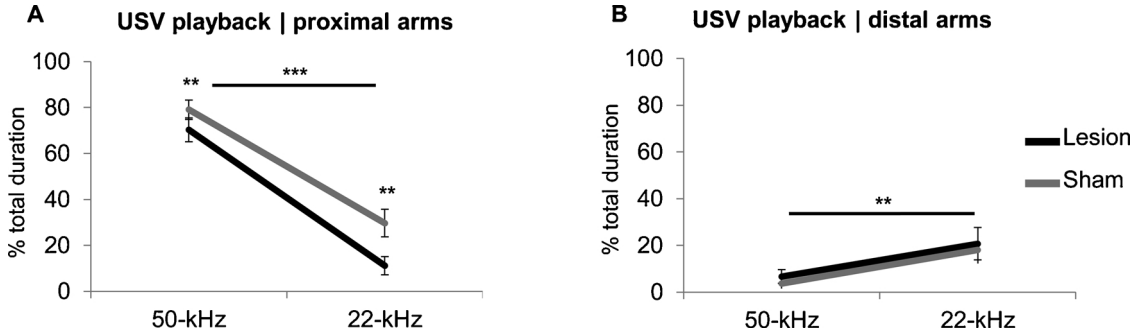

Fig. 4. BLA lesions reduced the time spent in the proximal arms during the first USV exposure session.
A: All rats spent more time in the proximal arms during 50-kHz USV playback, compared to 22-kHz USV playback. BLA lesions reduced the total duration spent in the proximal arms during both types of USV playback. B: All rats spent less time in the distal arms during the 50-kHz playback compared to 22-kHz playback, irrespective of the lesion. Only data obtained during test session 1 were analyzed. Mean ± SEM. ***$p < .001$, **$p < .01$, *$p < .05$, n.s.: not significant.

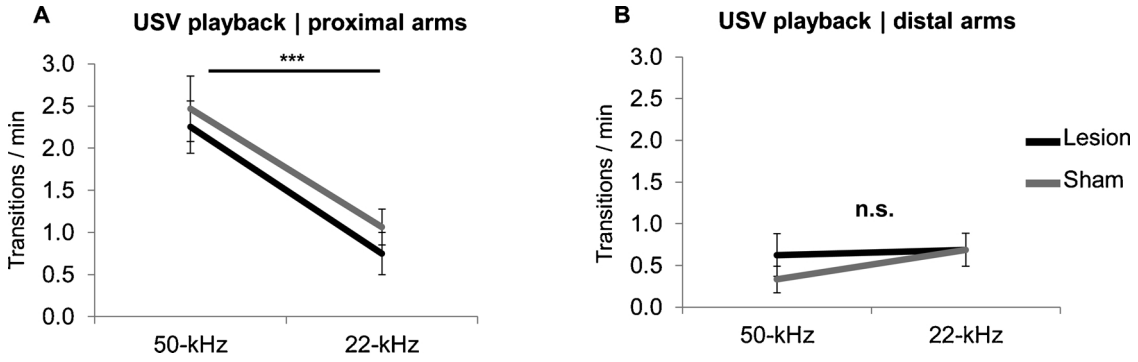

Fig. 5. BLA lesions reduced the number of transitions into the proximal arms during the first exposure to the USV stimuli.
A: All rats transitioned more often into the proximal arms during 50-kHz USV playback than during 22-kHz USV playback. B: A significant difference in time spent in the distal arms was not found during USV playback. Only data obtained during test session 1 were analyzed. Mean ± SEM. ***p < .001, *p < .05, ISI: inter-stimulus interval.

3.4. BLA lesions amplified immobilization in response to 22-kHz USVs

Next, we examined in detail whether 50-kHz and 22-kHz USV stimuli would induce approach, avoidance or immobilization behavior and whether this behavioral response pattern was modulated by BLA lesions during the first test session. Since our analyses have shown that rats spent less time in the vicinity of the speaker during playback of 22-kHz USVs, we included an analysis of the total duration of time spent in the center of the maze during the USV playback phase. We performed a mixed ANOVA with USV type (50- *vs*. 22-kHz USVs) as within-subjects factor and lesion (BLA lesion *vs*. sham) as between-subjects factor on data obtained during the first test session. All rats spent significantly more time in the center of the maze during playback of 22-kHz USVs compared to 50-kHz USV playback [$F(1,29) = 14.13$; $p < .01$; Fig. 6A], which also matches our subjective observation that numerous rats became immobile at the onset of 22-kHz USV playback. During the USV playback phase, rats with a BLA lesion spent a greater amount of time in the center of the maze compared to sham rats [$F(1,29) = 4.99$; $p < .05$; Fig. 6A] and this group difference was especially pronounced during 22-kHz USV playback compared to playback of 50-kHz USVs.

In addition, we performed a mixed ANOVA with arms (proximal *vs*. distal) as within-subjects factor and lesion (BLA-lesion *vs*. sham) as between-subjects factor on the total duration of time spent in the respective arms during 50-kHz and 22-kHz USV playback separately,

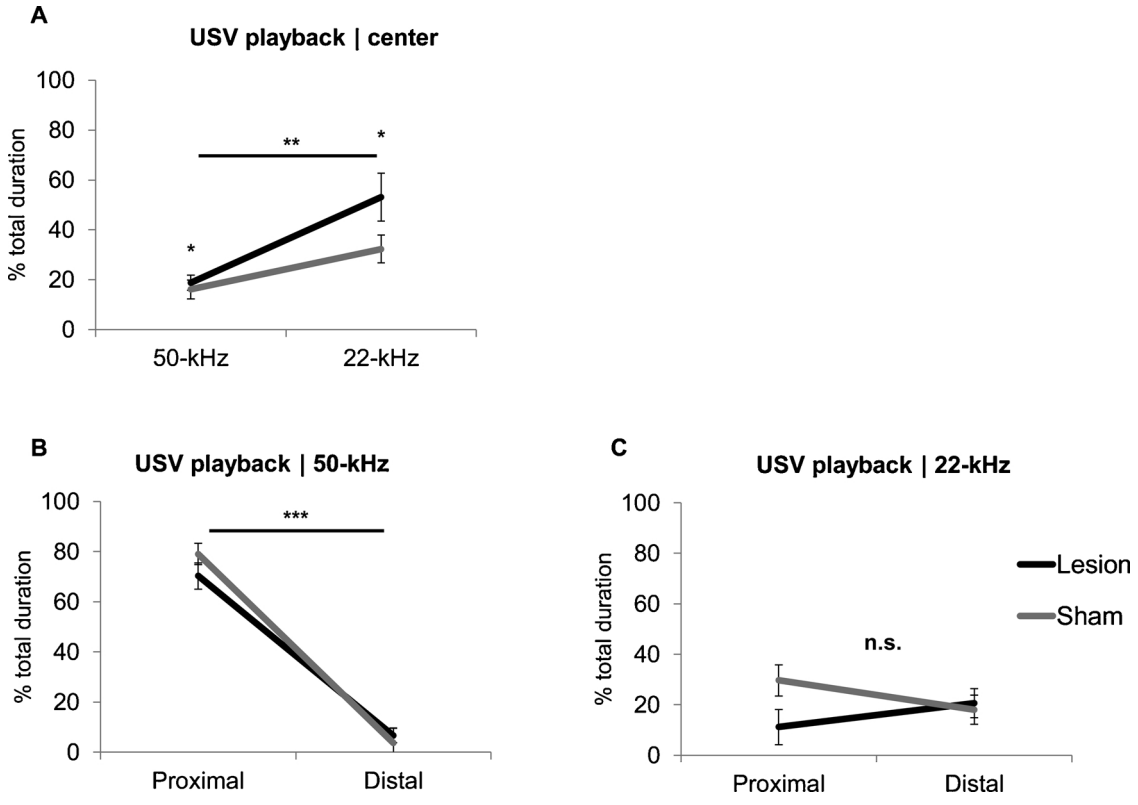

Fig. 6. Playback of 50-kHz USVs induced robust approach behavior in all rats.
A: During 22-kHz playback, all rats spent more time in the center of the maze compared to 50-kHz playback. Also the total duration in the center was higher for rats with BLA lesions. B: Direct comparison between the total duration spent in the proximal and the distal arms revealed that all rats spent significantly more time in the proximal arms during playback of 50-kHz USV playback, interpreted as approach behavior. C: During playback of 22-kHz the time spent in the proximal and distal arms was not significantly different, speaking against specific approach or avoidance behavior. Only data obtained during test session 1 were analyzed. Mean ± SEM. *** p < .001, ** p < .001, * p < .05, n.s.: not significant.

again on data obtained during the first test session. During playback of 50-kHz USVs, rats spent significantly more time in the proximal arms compared to the distal arms [$F(1,29) = 212.52$; $p < .001$; Fig. 6B], meaning that the rats robustly approached the speaker during 50-kHz USV playback. The amount of time spent in the proximal and distal arms was not significantly different during 22-kHz USV playback [$F(1,30) = 0.03$; $p < .05$; Fig. 6C], suggesting a lack of evidence for active avoidance of the 22-kHz USVs. In contrast to the previous analyses, statistically significant lesion effects on the behavioral response towards USV playback were not detected during the first test session.

3.5. BLA lesions did not affect motor functions or auditory perception

To exclude the possibility that the lesion effects on behavior could be explained by potential motor disabilities that might arise either from direct damage to the amygdala or from secondary lesion effects, we analyzed locomotor behavior in lesion and sham rats during the first test session. To this end, we performed a mixed ANOVA with USV type (50- *vs.* 22-kHz USVs) as within-subjects factor and lesion (BLA lesion *vs.* sham) as between-subjects factor on the total distance moved during USV playback. The distance moved was significantly higher for all rats during playback of 50-kHz USVs compared to 22-kHz USVs [$F(1,29) = 5.78$; $p < .05$] without a significant difference between the groups [$F(1,29) = 0.19$; $p > .05$; Fig. 7A]. Because of the lack of group differences, it is unlikely that the lesion effect on behavioral changes previously found during the USV playback phase was due to general motor impairments caused by the lesion. In addition, we wanted to exclude any impact of generalized fear on behavior in the BLA-lesioned rats that would cause the decreased total duration of time spend in the proximal arms (Fig. 4A) or the increased total duration spent in the center (Fig. 6A) during USV playback within the first test session. BLA-lesioned rats did not differ significantly from sham rats in the duration of time spent in the proximal arms or the center during all phases preceding the USV playback (data not shown). Thus, the observed group differences in behavior were exclusively present in relation to USV playback.

Lastly, we wanted to exclude the possibility that BLA lesions affected general auditory perception. Additional analyses revealed that rats spent less time in the proximal arms during playback of background noise than during the background ISI phase within the first test session [$F(1,30) = 13.15$; $p < .01$; Fig. 7B and C]. However, significant lesion effects on time spent in the proximal arms were not detected during the background noise or background ISI phase [$F(1,30) = 0.04$; $p > .05$]. Thus, all rats seemed to avoid the background noise, implying intact auditory perception of the noise stimulus in sham as well as lesion animals. Based on these results we assume that BLA-lesioned rats were able to perceive the acoustic stimuli similarly to sham animals, therefore we conclude that the effects of BLA lesions on the time spent in the proximal arms during USV playback (Fig. 4A) cannot be explained by deficits in auditory perception. Rather, the lesion effect on behavior seemed to be specific for the social aspect of USVs.

4. Discussion

In this study, we investigated the effects of BLA lesions on behavior towards 50-kHz and 22-kHz USV playback in an eight-arm radial maze. We found that the total duration spent in the proximal arms was longer for 50-kHz USV playback than for 22-kHz USV playback in all rats

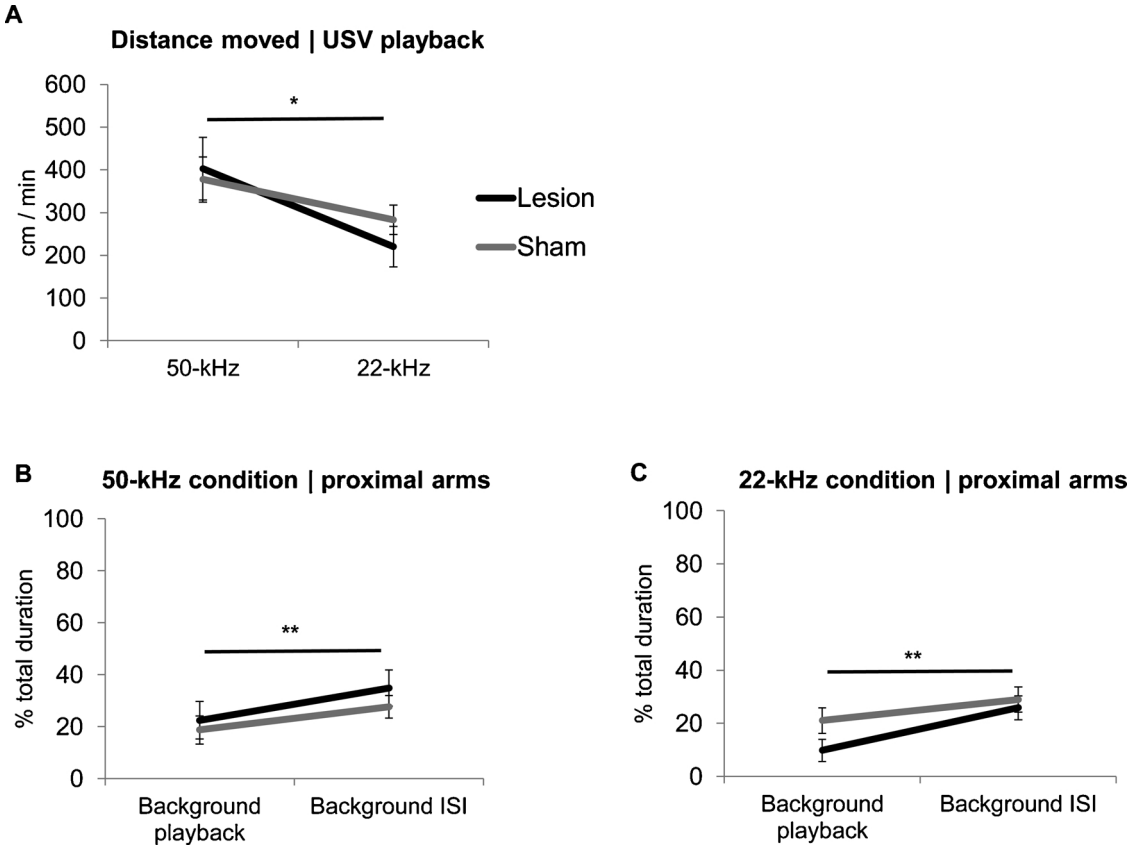

Fig. 7. BLA lesions did not affect general motor function or auditory processing.
A: All rats significantly increased their total distance moved during playback of 50-kHz USVs compared to 22-kHz USVs. However, significant differences were not detected between BLA-lesioned and sham rats. B and C: All rats spent less time in the proximal arms during playback of background noise compared to the subsequent ISI phase. There was no lesion effect on time spent in the proximal arms during both phases, which indicates intact responding to the auditory background noise stimulus in BLA-lesioned animals. Only data obtained during test session 1 were analyzed. Mean ± SEM. ***$p < .001$, **$p < .01$, *$p < .05$.

(Fig. 4A) and the total duration spent in the distal arms was longer for 22-kHz USV playback compared to 50-kHz USV playback (Fig. 4B). At the same time, rats were more immobile, staying longer on the center platform during 22-kHz compared to 50-kHz USV playback (Fig. 6A). In the proximal arms, rats with bilateral BLA lesions showed an attenuated response to both types of USV stimuli (Fig. 4A), especially to playback of 22-kHz USVs, whereas in the distal arms, a difference between the groups was not detected (Fig. 4B). In addition, rats with BLA lesions spent more time in the center of the maze compared to sham rats, an effect that was again most pronounced during playback of 22-kHz USVs (Fig. 6A). In summary and contrary to the behavior of the sham rats during 50-kHz USV playback, which could be identified as clear approach behavior, lesioned rats neither approached nor actively avoided the speaker during 22-kHz USV playback; instead, they spent more time in the center of the maze.

Consistent with previous reports [20,37], approach behavior to 50-kHz USV playback decreased with repeated stimulus exposure in all rats. Accordingly, the lesion effects on behavior were only evident during the first exposure to the USV stimuli. The difference between lesioned and sham animals was lost together with the effect of USV playback on behavior during subsequent exposures. Based on that finding, the BLA seems to motivate certain types of behavior towards salient stimuli, but is not essential for the habituation towards these stimuli. Lesions of the BLA did not impair general auditory processing or motor functions, since all rats responded to playback of background noise, which implies intact auditory perception of the noise stimulus, and lesioned rats showed intact motor behavior similar to sham rats. In addition, lesion effects were specific to USV playback and absent while rats were left to explore the maze before the occurrence of USV playback, arguing against generalized fear of BLA-lesioned rats during maze exploration. Therefore, we conclude that the effects of BLA lesions on behavior were specific to the social nature of the USVs. Overall, our findings are consistent with our hypothesis that the BLA plays a role in motivating approach behavior towards auditory stimuli with social significance.

USVs are used by rats to communicate and transmit contextual and affective information and can influence the behavior of conspecifics [38]. The mere playback of USVs can modulate the behavior of rats, for example by increasing or decreasing locomotion [12,15], induction of freezing [39] and approach behavior [12], or even affect complex behaviors such as mating and decision making [18]. In line with this, playback of 50-kHz USVs has been shown to elicit increased activation and a rise in dopamine in the nucleus accumbens, a core region within the reward circuitry of the brain [19,20]. These findings suggest that USVs have motivating properties and therefore could reinforce behavior contingent on USV emission. Here, we found that rats with BLA lesions showed an attenuated behavioral response towards USV playback, therefore we suggest that the BLA plays a role in mediating the reinforcing properties of USVs.

It could be argued that the increased time spent in the center of the maze by BLA-lesioned rats is a consequence of increased anxiety. However, this interpretation is inconsistent with the anxiolytic effects of whole amygdala lesions, BLA lesions or pharmacological inactivation of the BLA [40–42]. In line with this, behavioral differences between rats with a BLA lesion and sham rats could not be detected in the experimental phases preceding USV playback, implying that the decreased time spent close to the USV-emitting speakers and the increased time spent in the center by animals with a BLA lesion was not due to a general increase in anxiety. Also it is of note that we did not find active avoidance behavior in response to playback of 22-kHz USV stimuli. The lack of overall increases in anxiety after BLA lesions in our study and the fact that in other studies lesions of the BLA dampen, rather than induce anxiety, reduces the likelihood that the increased time spent in the center of the maze is due to increased anxiety towards the USV playback. Alternatively, we suggest that the effect of BLA lesions on the time spent in the center of the maze could be caused by the USVs losing their incentive salience.

Besides its significant role in anxiety, the BLA is a region responsible for a wide variety of processes and BLA lesions have been shown to alter working memory [43], reward learning [44], decision making [45] and emotional expressions [46]. It has been suggested that the BLA is responsible for forming cue-reward associations [47] and lesions of the BLA prevented the response to conditioned cues linked to rewarding behavior [48,49]. As mentioned, a reduced responsiveness of BLA-lesioned rats to USV playback might indicate a loss of reinforcing value of the USVs. Although 22-kHz USVs are emitted in aversive situations and therefore not considered as 'classical' reinforcers (as they are supposed to transmit information about potential threats), their social-communicative function might possess reinforcing value on its own. Neurons in the BLA encode stimuli with both positive and negative value and there is an ongoing debate about whether these neurons can be anatomically segregated or not. In one study, neurons in the anterior portion of the BLA responded preferably to aversive events, whereas neurons in the posterior BLA fired during reward [50]. In our study, lesions covered primarily the anterior BLA (Fig. 2), which could lead to a reduced sensitivity towards aversive stimuli such as 22-kHz USVs. This in turn might either translate into less anxious behavior causing more behavioral exploration of the maze during 22-kHz USV playback, or less anxious behavior causing a lack of interest in the source of 22-kHz USV playback. The latter explanation would suit our findings of reduced behavioral responses during USV playback following a BLA lesion. However, the increased immobilization response towards 22-kHz USV playback observed in BLA-lesioned rats might still reflect anxious behavior, pointing towards a more complex scenario. In addition, the anatomical segregation of neurons encoding different valences within the anterior and posterior BLA could not be replicated in a more recent study [51], meaning that neurons firing during aversive and rewarding experiences might as well be scattered throughout the entire BLA.

It is unclear whether USVs should be considered innate primary reinforcers, or whether USVs gain conditioned incentive value through association with appetitive or aversive events [39,52,53]. Our observed transience of the behavioral responses to USV playback is inconsistent with the assumption that listening to USVs is inherently rewarding, or aversive, respectively, since there is no parsimonious explanation for the rapid loss of the alleged primary hedonic nature of the USV stimuli. By contrast, our finding is more in line with the view that USVs act as cues associated with an affective social consequence. The lack of such a consequence in an artificial playback setting, *i.e.* the absence of a social encounter after USV playback, might then lead to rapid extinction of the previously acquired associations between USVs and their social corollaries. The BLA might modulate these learning and extinction phenomena, but future research is necessary for further clarification.

The results from our experiment provide additional insights into the psychological and neural basis of prosocial behavior [54]. Our group has previously shown that rats prefer mutual food rewards, benefitting themselves and a conspecific, to food rewards delivered only to the respective rat, but not to its conspecific in a rodent prosocial choice task [55]. In a recent study, we found that mutual-reward preferences disappeared after bilateral BLA-lesions [35]. Integrating this finding with our current results, the abolishment of mutual-reward preferences after BLA lesions can be tentatively explained by the hypothesis that the BLA is important for spatially approaching the mutual-reward site in the prosocial choice maze. The BLA might support this approach behavior by processing the incentive value of social signals, such as USVs, that are emitted by both rats during task performance and that come to orchestrate their social choice behavior [18,54].

4.1. Conclusion

In summary, we found that lesions of the BLA reduced the behavioral response towards USV stimuli, likely because the USVs had a diminished incentive value. Our results provide novel information

about the role of the BLA in social communication of rats, adding to the field of the neurobiological basis of social cognition.

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Declaration of Competing Interest

There was no conflict of interest.

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