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Using bedding in a test environment critically affects 50-kHz ultrasonic vocalizations in laboratory rats

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ABSTRACT

Rats utter distinct classes of ultrasonic vocalizations depending on their developmental stage, current state, and situational factors. One class, comprising the so-called 50-kHz calls, is typical for situations where rats are anticipating or actually experiencing rewarding stimuli, like being tickled by an experimenter, or when treated with drugs of abuse, such as the psychostimulant amphetamine. Furthermore, rats emit 50-kHz calls when exposed to a clean housing cage. Here, we show that such vocalization effects can depend on subtle details of the testing situation, namely the presence of fresh rodent bedding. Actually, we found that adult males vocalize more in bedded cages than in bare ones. Also, two experiments showed that adult rats emitted more 50-kHz calls when tickled on fresh bedding. Furthermore, ip amphetamine led to more 50-kHz vocalization in activity boxes containing such bedding as compared to bare ones. The analysis of psychomotor activation did not yield such group differences in case of locomotion and centre time, except for rearing duration in rats tested on bedding. Also, the temporal profile of vocalization did not parallel that of behavioural activation, since the effects on vocalization peaked and started to decline again before those of psychomotor activation. Therefore, 50-kHz calls are not a simple correlate of psychomotor activation. A final experiment with a choice procedure showed that rats prefer bedded conditions. Overall, we assume that bedded environments induce a positive affective state, which increases the likelihood of 50-kHz calling. Based on these findings, we recommend that contextual factors, like bedding, should receive more research attention, since they can apparently decrease the aversiveness of a testing situation. Also, we recommend to more routinely measure rat ultrasonic vocalization, especially when studying emotion and motivation, since this analysis can provide information about the subject's status, which may not be detected in its visible behaviour.

1. Introduction

Although emotions in animals were demonstrated rather early by the pioneering work of Charles Darwin (1872/1998), their study has not become an essential topic in science until about the 70ties of the last century (Panksepp, 1998). Nowadays, however, many experts agree on the assertion that not only humans, but also other higher-order animals, especially mammals, are endowed with basic emotional mechanisms, since they show species-specific emotional responses in behaviour (like freezing, avoidance or approach), facial expressions, and various physiological reactions, which are similar or homologous to those of humans in comparable situations. Also, some researchers assume that animals, for example rats, may have basic and emotion-specific feeling states, that is, the feature, which humans often consider as most characteristic for their subjective emotional experiences (Panksepp, 1998). Similar to man, the study of animal emotion is largely based on facial expressions, vocal signs, and several types of motor behaviour (like approach, startle, freezing etc.). Animal research, however, is not endued with the 'gold standard' of human research, namely, measures of written or verbal self-report (like questionnaires), but it has been suggested that the analysis of vocal signs in mammals, like in monkeys (Seyfarth and Cheney, 2003), may partly fill this gap, since they may also serve as a measure of their subjective state.

In rats, studying vocalizations may also be useful when it comes to their putative emotional states. They, like several other rodents, emit various types of calls. Importantly, their majority occur in the ultrasonic range, which, was first described in 1954 (Anderson, 1954). Meanwhile, two classes of calls have been defined in adult rats, namely so-called 22- and 50-kHz calls (Brudzynski, 2005). The former has already contributed substantially to research on negative emotions and their pharmacology, especially anxiety and fear (Borta et al., 2006; Wöhr et al., 2005), since they are often considered as "distress", "threat", or "alarm" calls (Covington and Miczek, 2003; Litvin et al., 2007; Sanchez, 2003). In contrast, 50-kHz calls are typical for situations with a positive emotional valence, that is, where rats

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actually experience or expect various kinds of rewards like juvenile play, tickling by a skilled experimenter (also termed hetero-species play behaviour), mating, food, rewarding brain stimulation, or certain drugs of abuse, like amphetamine or morphine (Barfield and Thomas, 1986; Barfield et al., 1979; Bialy et al., 2000; Burgdorf and Panksepp, 2001; Burgdorf et al., 2000, 2001a, 2001b, 2007; Knutson et al., 1998, 1999, 2002; Panksepp and Burgdorf, 2000; Sales, 1972; Schwarting et al., 2007; Thompson et al., 2006; White et al., 1990; Wintink and Brudzynski, 2001; Wöhr et al., 2009). Furthermore, we have repeatedly shown, that tickling elicits 50-kHz calls also in adult rats (Schwarting et al., 2007; Wöhr et al., 2009), albeit at a lower rate compared to juvenile rats during play age (Mällo et al., 2007). Jaak Panksepp (2005) argued that such 50-kHz calls may constitute an ancient homolog of human laughter. Similar to laughter (Provine, 2000), 50-kHz calls may index a positive affective state, and/or they may serve as a social signal (Wöhr and Schwarting, 2007; Wöhr et al., 2008). Thus, it has repeatedly been reported that rats emit 50-kHz calls when exposed singly to a test arena or a clean home cage (termed "cage test") containing bedding (Brudzynski and Pniak, 2002; McGinnis and Vakulenko, 2003) with no change in call rate when tested on 4 consecutive days (Schwarting et al., 2007). Further studies showed that the cage mate, which remained alone in the home cage, emitted even more calls than the one transferred to another cage (Wöhr et al., 2008). These data show that 50-kHz calling is not necessarily a response to the actual presence of pleasurable or social stimuli, but also their anticipation (here expected reestablishment of social contact), the likelihood of which may be promoted by social signalling, i.e. approach-eliciting features of these calls (Wöhr and Schwarting, 2007).

Here, we show that the occurrence of 50-kHz calls can critically depend on certain features of the testing situation, namely whether rats are tested in cages or apparatuses with or without commercial rodent bedding. In a series of experiments, we tested how the presence of bedding affects ultrasonic vocalization, namely in the cage test, to being tickled, and to systemic amphetamine. In a final experiment, we asked whether rats also prefer environments with bedding by exposing them to a choice apparatus, where they could chose between environments with or without bedding.

2. Materials and methods

2.1. Experiment I: cage test

2.1.1. Animals and bedding

Twenty-five naive male Wistar rats (Harlan Winkelmann GmbH) were used that weighed 185–218 g and were about 1.5 months old on the first testing day. They were housed in groups of 4–5 in type-IV cages (550×330×200 mm) in an animal room with a 12 h light:12 h dark photoperiod (lights on 07:00 am). Rat chow (Altromin GmbH, Lage, Germany) and tap water were provided ad libitum. Before testing, rats were handled for 3 days. Throughout this work, we only used one kind of bedding, namely Tapvei® which is a dust-free bedding type consisting of small chipped pieces (wood chips roughly 5×5×1 mm in size) of Aspen wood (indulab ag, Gams, Switzerland).

2.1.2. Test procedure

Animals were randomly assigned to two groups. They were tested in clean type-III Makrolon cages (378×217×180 mm), one group ($n=13$) in cages that contained a layer (about 2 cm thick) of fresh bedding and the other ($n=12$) in empty cages of the same size.

For testing (10 min), the rats were removed from the group cage and placed in their fresh testing cage with steel grid cover. Then, the cage was carried to the adjacent ultrasonic lab, where it was placed on a small testing desk. There was no other rat present in the lab, which was illuminated by white light of about 2 lx. The ultrasonic microphone was mounted centrally at about 35 cm above the cage floor. Additionally, a video camera connected with a DVD recorder was positioned at a longitudinal side of the cage to record visible behaviour, which was evaluated offline by observation of rearing and locomotor activity (according to Schwarting et al., 2007). Rearing was quantified as the number of times the animal reared on its hind legs. For locomotor activity, the cage was divided into two virtual halves, and the number of times the rat crossed this line was counted. After testing, the animal was brought back to the animal room and placed back into its group cage. Tests were always done with fresh cages (and bedding) and were repeated on two consecutive days, with testing order changed randomly between days.

2.2. Experiment II: repeated cage test

2.2.1. Animals

Twelve male Wistar rats were used when they weighed 407–490 g (about 4 months old) on the first testing day. They had previously been used in Exp. I and were housed and handled under the same conditions.

2.2.2. Test procedure

Technical details were the same as in Experiment I, except that all animals were tested in clean cages without bedding on six consecutive days. In the subsequent analysis, data from only the 1st and the 6th day were used.

2.3. Experiment III: tickle test A

2.3.1. Animals

Twelve experienced male Wistar rats were used. They weighed between 284 and 359 g (about 2.5 months old) on the first testing day and were housed in groups of six in type-IV Macrolon cages and under the same conditions as in Experiment I. Rats were handled for 3 days before testing.

2.3.2. Test procedure

Animals were randomly assigned to two groups ($n=6$ each), and one group was tickled in clean type-III Makrolon cages that contained fresh bedding (a layer of about 2 cm), the other group was tickled in clean but empty cages of the same size. All rats were tickled on two consecutive days, with their testing order changing randomly from day to day. For testing, a given rat was removed from its group cage and placed into the testing cage without a cover. Then, the cage was carried to the adjacent ultrasonic lab, where it was placed on a small testing desk. There, no other rat was present, and the room was illuminated by red light of about 28 lx. The ultrasonic microphone was mounted centrally at about 35 cm above the cage floor. The experimenter sat down in front of the cage and manipulated the rat with the left hand following a standardized procedure (10 min), which contained different components, namely "neck tickle", "belly tickle", "push and drill", "full back", "tail chase", "hand chase" and "grab and tickle" (for details see Schwarting et al., 2007). Each component lasted 30 s; furthermore, six 30-s breaks were interspersed at 0, 60, 150, 300, 420 and 570 s. During these breaks, the experimenter's hand remained passively inside the cage. After testing, the animal was brought back to the animal room and placed into its group cage.

2.4. Experiment III: tickle test B

Since the data of tickle test A were not fully conclusive, we decided to perform an additional experiment, where we attempted to optimize several factors: For one, we used a slightly larger sample ($n=16$; 331–440 g and 2.5–3 months at the start of tickling). Secondly, we initially performed a cage test (methods see section 2.1.2.) to screen for individual differences in call rates (see also

Schwarting et al., 2007). Based on the individual call numbers in this test, which ranged between 0 and 140 calls in 5 min, we matched our 16 rats to two treatment groups with similar basal call rates (group bedding: 20.87 ± 5.94, group no bedding: 34.00 ± 15.84, mean ± SEM; 2-tailed Mann–Whitney *U* test between groups: $p = .645$). Also, we enhanced the subsequent tickling experience to 5 consecutive days (10 min each) and tested under red light of 10 lx. The reason to use more days was that rats seem to become accustomed to the procedure with days, that is, they initially also emit 22-kHz calls, which are presumably aversive (Schwarting et al., 2007; Wöhr et al., 2009), and which become less frequent with repeated tickling experience. Therefore, we only analyzed vocalizations from the final (i.e. 5th) treatment day. Also, we slightly modified our tickling protocol, that is, we deleted the components "tail chase" and "full back", since they appeared least efficient to elicit 50-kHz calls. Also, we replaced "grab and tickle" by "flip over", where the experimenter picks the animal and flips it on its back. This component is also effective to elicit 50-kHz calls and has face validity to rat rough-and-tumble play, namely pinning. Identical to tickle test A, each component lasted 30 s and was applied between 2 and 4 times on every test day. Also, six "breaks" were performed again at irregular time points.

2.5. Experiment IV: amphetamine

2.5.1. Animals

Twenty-four naive male Wistar rats were used that weighed 209–251 g (1.5–2 months) on the first testing day. They were housed in groups of three in type-IV cages under the same conditions as in Exp. I and were handled for 3 days before testing. Also, we exposed all rats once for 5 min to a clean type-III housing cage containing fresh bedding (cage test) to test for individual differences in call rates according to our previous work (Schwarting et al., 2007). Based on the individual call numbers in this test, which ranged between 0 and 90 calls in 5 min, we matched our 24 rats to two subsequent treatment groups with similar basal call rates (group 1: 5.18 ± 1.87, group 2: 4.43 ± 1.46, mean ± SEM; 2-tailed Mann–Whitney *U* test between groups: $p = .810$).

2.5.2. Test procedure

Rats were tested on three consecutive days for 45 min each in an acrylic activity box (40 × 40 × 40 cm) with a floor made of plastic. Two photobeam sensor rings and an automated activity monitoring system (TruScan™, Photobeam Sensor-E63-22; Coulbourn Instruments; USA) were used to obtain the following measures: A) locomotion (distance travelled per time), B) centre time (in s per min) and C) rearing (in terms of frequency and time). The box was situated in the ultrasonic lab, which was illuminated by red light of about 28 lx. For call recording, the ultrasonic microphone was mounted centrally at 50 cm above the floor of the box. One group (group 1) was tested with fresh bedding (on the floor) and the other (group 2) without. On the first day, animals were tested without injection. On day 2, a 0.9% saline solution was injected (ip) just before testing, and on day 3, rats were injected with D-amphetamine (2.5 mg/kg body weight ip; Sigma). Testing order of rats changed randomly from day to day.

2.6. Experiment V: choice test

2.6.1. Animals

Twelve male Wistar rats were used, which weighed between 317 and 389 g (about 2.5 months old) on the first testing day. They were housed under the same conditions as in Experiment I and were handled for 3 days before testing.

2.6.2. Test procedure

An apparatus with two compartments (each 23 × 35 × 35.5 cm; *L* × *W* × *H*) connected by a central alley (11 × 9 × 35.5 cm) was used. One compartment contained fresh bedding, whereas the other and the central alley remained unbedded. The apparatus was placed on a desk in the ultrasonic lab, which was illuminated by red light of about 28 lx. For call recording, an ultrasonic microphone was mounted above the centre of each compartment at 40 cm above its floor. A video camera connected with a DVD recorder was positioned above the box to record the time spent in each compartment. For testing (20 min), a given rat was removed from its group cage and placed into the central alley. Half of the animals were tested with bedding in the left and the other half with bedding in the right compartment.

2.7. Recording and analysis of ultrasonic vocalization

An UltraSoundGate Condenser Microphone (CM 16) sensitive to frequencies between 10 and 120 kHz with a flat frequency response between 15 and 30 kHz (±6 dB) and between 40 and 70 kHz (±12 dB) was used. It was connected via an Avisoft UltraSoundGate 416 USB audio device (Avisoft Bioacoustics, Berlin, Germany) to a personal computer, where acoustic data were displayed in real time by Avisoft Recorder (Avisoft Bioacoustics), which recorded with a sampling rate of 214 285 Hz in 16 bit format.

For acoustical analysis, recordings were transferred to SASLab Pro (version 4.3; and 4.52; Avisoft Bioacoustics) and a fast Fourier transform was conducted (512 FFT-length, 100% frame, Hamming window and 75% time window overlap). Spectrograms were produced at 488 Hz of frequency resolution and 0.512 ms of time resolution. Call detection was provided interactively by an experienced user, who marked the calls manually.

2.8. Statistical analysis

Data are presented as mean with SEM. To test for statistical differences, we used non-parametric statistics (Mann–Whitney *U* test, Wilcoxon test; 2-tailed) in case of USV, since call rates often did not fulfil the criterion of normal distribution. All other behavioural data were tested parametrically, that is, with paired or unpaired *t*-tests (2-tailed), or with ANOVA for repeated measures. *p*-values of less than .05 were considered as significant.

3. Results

3.1. Experiment I: cage test

In this test, rats were simply exposed singly to a clean housing cage. This test (Fig. 1) revealed differences in 50-kHz calling dependent on the bedding condition. Rats tested in a bedded cage emitted more 50-kHz calls than those tested in a bare one. This effect was observed on both test days (day 1: $p = .001$, day 2: $p = .015$; two-tailed), with no difference between days. In contrast, behavioural activity, that is, the number of rearings or locomotor activity did not differ between groups (*p*-values between .170 and .819), and declined from day 1 to day 2, except for rearing in the group tested on bedding.

3.2. Experiment II: repeated cage test

Experiment I showed an influence of bedding on the call behaviour of rats. We wondered if the higher call rate in rats tested in bedded cages is due to familiarity with this environmental stimulus. Therefore, in a second experiment, we exposed the animals repeatedly to an unbedded cage to test whether the animals increase call rate when they become familiar to the test environment.

In this test, 50-kHz call rate did not change from the 1st to the last testing day (day 1 vs. day 6: $p = .654$; Fig. 2), whereas behavioural activity habituated, that is rearing ($p < .001$) and locomotor activity ($p = .002$) was lower on day 6 as compared to day 1. Thus, increased familiarity with the unbedded cage did not induce calling.

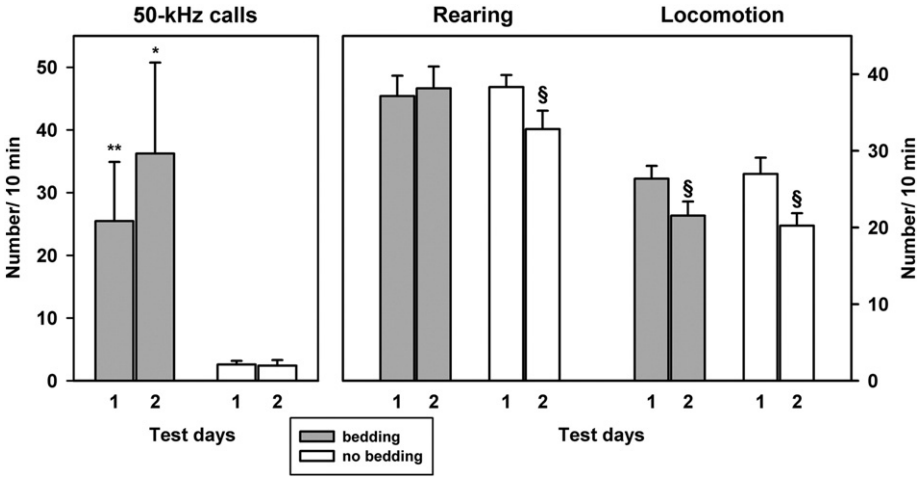

Fig. 1. The number (counts/10 min; mean + SEM) of 50-kHz calls (left), rearing (middle), and locomotor activity (right) during housing cage tests (10 min each) performed on two consecutive days. Two groups of rats were tested singly either in clean housing cages with fresh bedding (gray bars; $n = 13$), or in identical cages without bedding (open bars; $n = 12$). Symbols depict differences between groups with $^{*}p < .05$, $^{**}p < .01$ and differences between test days with § $p < .05$.

3.3. Experiment III: tickle test A

All rats tickled in bedded cages emitted at least some 50-kHz calls, with a range of 4–154 calls on day 1 (mean ± SEM: 60.17 ± 23.97), and 4–181 calls on day 2 (67.67 ± 33.35). In the group tested in unbedded cages, two rats did not emit 50-kHz calls on day 1 and two other rats did not vocalize in such a way on day 2. In the others, call rate ranges were 3–41 on day 1 and 1–13 on day 2. The group mean (± SEM) in case of the unbedded condition was 8.50 ± 6.54 on day 1 and 2.83±2.06 on day 2. On both days, the statistical analysis yielded more 50-kHz calls for the group tested in bedded cages (day 1: $p = .024$, day 2: $p = .009$).

Regarding 22-kHz calls, three rats in the unbedded condition emitted such vocalizations (day 1: range 46–152, mean ± SEM 48.50 ± 25.66; day 2: range 42–96, mean ± SEM 37.67 ± 18.45), as compared to only one rat in the bedded condition. This animal, however, had the highest call rate (day 1: 345 calls, day 2: 210 calls). Therefore, the statistical analysis did not yield differences between groups (day 1: $p = .545$, day 2: $p = .545$).

3.4. Experiment III: tickle test B

During the test on the fifth day of tickling, all subjects emitted at least some ultrasonic calls. In the group tickled on bedding, the number of 50-kHz calls (146.38 ± 94.95) was higher than that tested without bedding (16.62 ± 6.03; 2-tailed, $p = .022$). During the 10-min test, the individual numbers ranged between 13 and 800 calls (with bedding) and between 1 and 53 calls (without bedding). Their mean frequencies ranged between 41 and 79 kHz and did not differ between groups. No typical 22-kHz calls (mean frequency around 22-kHz, long call duration), but some unusual low-frequency calls were observed individually ranging between 0 and 6 calls in 10 min. These calls lasted between 10 and 47 ms and had mean frequencies of 23 to 31 kHz. Their number did not differ between groups.

3.5. Experiment IV: amphetamine

3.5.1. Visible behaviour

In general, locomotor activity (Fig. 3) and rearing behaviour (Fig. 4) showed the typical test patterns in the activity box: Under undrugged test conditions (no injection, saline), they were highest during the initial 5 min and rapidly declined thereafter (factors of time, p-values <.001). The activating effect of amphetamine treatment on locomotion and rearing became observable around 5–10 min after injection and outlasted the 45 min period of testing.

Regarding test conditions with or without bedding, there were no significant differences in locomotion or centre time (Fig. 3) in any of the three test phases (factor group, all p-values >.05), nor were there

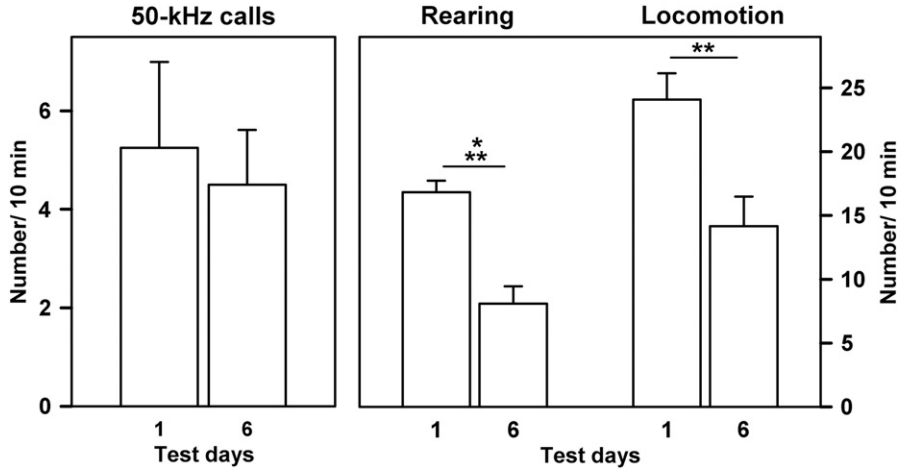

Fig. 2. The number (counts/10 min; mean + SEM) of 50-kHz calls (left), rearing (middle), and locomotor activity (right) during the 1st and the 6th test in a cage test (10 min each). All rats were tested in clean housing cages without bedding (open bars; $n = 12$). Differences between test days: $^{**}p < .01$, $^{***}p < .001$.

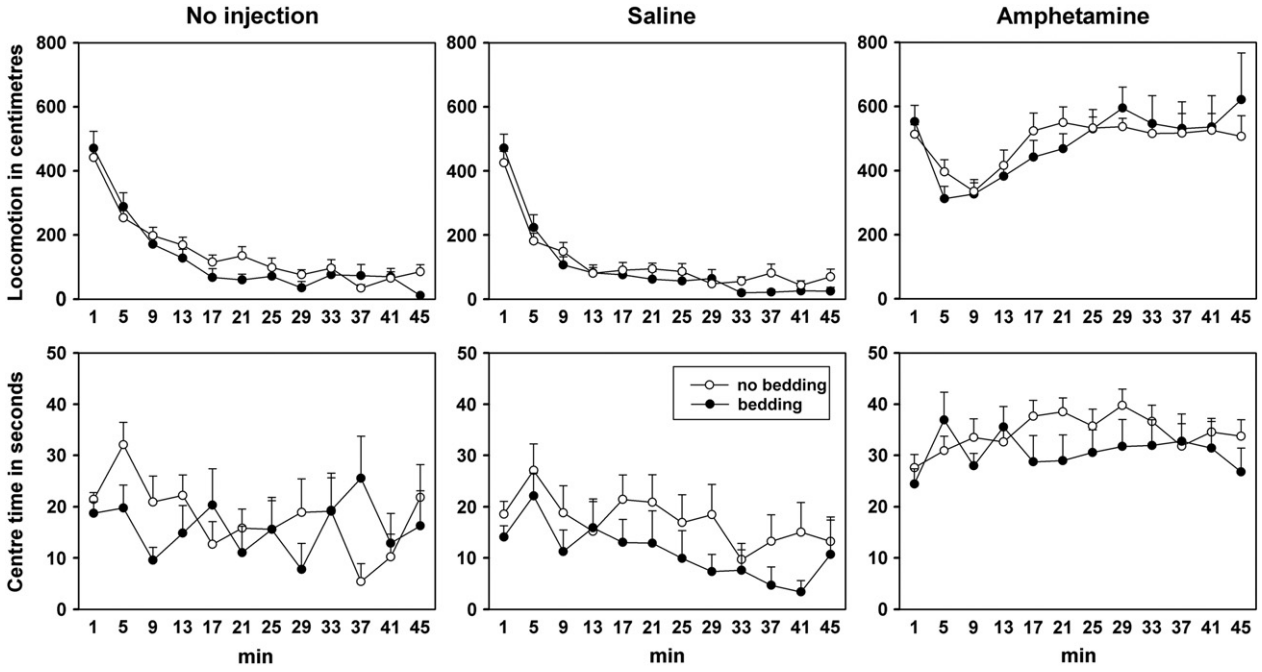

Fig. 3. Locomotor activity and centre time in the activity box. Rats were tested either without any injection, an ip injection of saline, or an ip injection with a dose of 2.5 mg/kg of amphetamine. Each test lasted 45 min, and behaviour was monitored every 4th minute. Locomotion (top) was expressed as centimetres travelled per respective minute (mean + SEM). Centre time (bottom) was measured in s per min (mean + SEM). One group of rats ($n = 12$) was tested in boxes with bedding on the floor (full symbols), the other group ($n = 12$) in boxes with bare plastic floors (open symbols).

interactions between the factors group and time (p-values >.05). In contrast, group differences were found in case of rearing behaviour (Fig. 4): The time spent rearing under amphetamine was higher in the group tested with bedding (factor group: $p = .033$) as compared to that without bedding. Comparisons of single time points yielded significant increases at 21 (2-tailed t-test: $p = .003$) and 25 min ($p = .017$) after injection, but not before or after (all p-values >.05). The number of rearings descriptively showed similar patterns, but there were no statistical group differences under amphetamine (factor group: $p = .113$). Also, there were no group differences in rearing in the preceding no-drug test phases.

3.5.2. Ultrasonic vocalization

During the two no-drug test phases, there were almost no 50-kHz calls (Fig. 5A) except for the initial minutes of the test periods. In the 1st test, i.e. without prior injection, the total number of these calls in

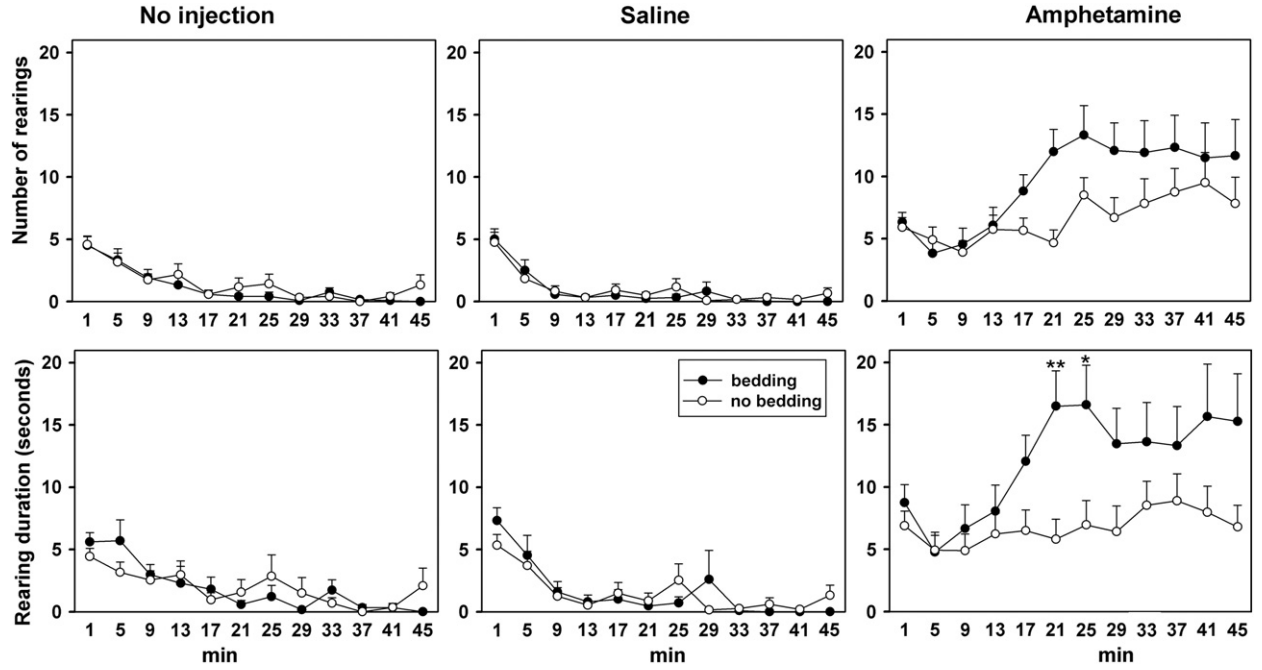

Fig. 4. Rearing behaviour in the activity box. Rats were tested either without any injection, an ip injection of saline, or an ip injection with a dose of 2.5 mg/kg of amphetamine. Each test lasted 45 min, and behaviour was monitored every 4th minute. Rearing was measured in terms of frequency (number, top) and time (bottom; in s per min; mean + SEM). One group of rats ($n = 12$) was tested in boxes with bedding on the floor (full symbols), the other group ($n = 12$) in boxes with bare plastic floors (open symbols). Symbols depict differences between groups: $^{*}p < .05$, $^{**}p < .01$.

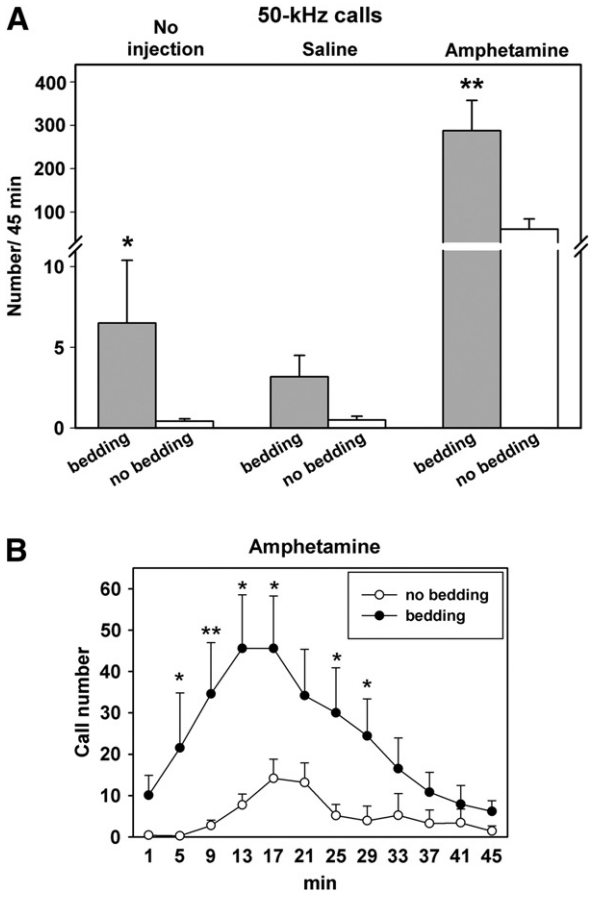

Fig. 5. Ultrasonic vocalization in the activity box. Rats were tested either without any injection, an ip injection of saline, or an ip injection with a dose of 2.5 mg/kg of amphetamine. Each test lasted 45 min. (A) shows the total number of 50-kHz calls (mean + SEM) during these three tests (45 min each), whereas (B) shows the time course of the amphetamine data (50-kHz calls) monitored every 4th minute. Filled bars and symbols stand for rats tested in the bedded activity box, whereas open ones stand for those tested in bare boxes. Asterisks depict differences between groups: $^{*}p<.05$, $^{**}p<.01$.

45 min was higher in the bedded than the non-bedded condition ($p=.024$), whereas there was no significant difference in the test after saline injection ($p=.089$). Compared to these no-drug tests, numerous 50-kHz calls were emitted when rats were treated with amphetamine, and these were more frequent in case of the bedded condition ($p=.003$ vs. no bedding for the total 45-min period). All twelve rats in the bedded condition called, and the sum of these calls over the twelve time points of testing ranged between 21 and 645 calls. In the group tested without bedding, only 8 out of 12 rats emitted such calls ranging between 1 and 287 calls. Individual differences in 50-kHz call rates cannot account for the group effects, since they had been matched for such patterns by the preceding cage test. Also, there were no substantial correlations between call numbers in the cage test and those under amphetamine (data not shown).

When analyzing the time course of these calls (Fig. 5B), we found that call activity peaked around 13–21 min after injection. Furthermore, rats tested on bedding called more than rats without bedding during min 5–17 and 25–29 (p-values between .039 and .007).

3.6. Experiment V: choice test

Rats preferred bedding, since they spent most of the time in the bedded compartment ($T=2.564$, $p=.026$, two-tailed; Fig. 6). Additionally, they emitted 50-kHz calls, and most of these calls were emitted when in the bedded compartment, where call number ranged between 2 and 84 calls. In the other compartment without bedding, 9 out of the 12 animals emitted calls ranging between a total of 1 and 16. Statistically, call number was significantly higher in the bedded side both, in terms of total call number (data not shown, $p<.001$) and also when calculating calls per time spent in a given compartment (bedded versus unbedded side: $p<001$, two-tailed). 22-kHz calls were only rarely observed and there was no indication for differences between compartments (data not shown).

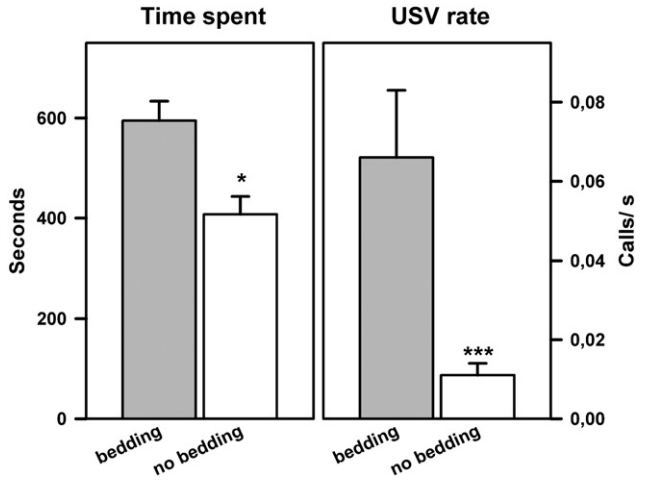

Fig. 6. Compartment preference and ultrasonic vocalization in a choice test. Rats (n = 12) were exposed for 20 min to an unfamiliar choice apparatus consisting of two equal-sized compartments connected by a central alley. In one compartment the floor remained bare, whereas it was covered with bedding in the other one. The rats showed a preference for the bedded compartment, since they spent most of the time there ($^{*}p<.05$). Also, they emitted significantly more 50-kHz calls when in the compartment with bedding (call number is expressed per time spent in s; $^{***}p<.001$).

4. Discussion

In a series of experiments in male Wistar rats, we have shown that the emission of 50-kHz calls is strongly dependent on environmental factors of the testing situation, since calls, a) when exposed singly to a housing cage, b) in response to being tickled by an experimenter, or c) when stimulated with amphetamine, were more frequent, if the animals were tested in an apparatus containing fresh bedding as compared to a bare one. Furthermore, we showed that rats preferred a bedded environment when given a choice, which was paralleled by more 50-kHz calls.

4.1. Cage test

Previously, we had found that rats exposed singly to a clean housing cage emit some 50-kHz calls, irrespective of whether this cage is bedded with fresh or soiled bedding (Schwarting et al., 2007; Wöhr et al., 2008). This finding is replicated here in case of fresh bedding and extended by another important aspect, namely that calling is clearly reduced if bedding is left away (Exp. I), and this effect was not reduced despite six repeated days of exposure to a bare cage (Exp. II). Therefore, increased familiarity with the bare testing situation was not sufficient to induce vocalization. Furthermore, the call differences between bedded and non-bedded testing conditions were not paralleled by gross differences in locomotion and rearing. Therefore, one can assume that 50-kHz calling is not a simple correlate of behavioural activation (see also Schwarting et al., 2007), at least not with respect to the measures taken here.

According to previous work (Schwarting et al., 2007), we assume that such 50-kHz calls, which rats otherwise emit during social

interactions, like juvenile play and reproductive behaviour, or when exposed singly to a housing cage (Barfield et al., 1979; Bialy et al., 2000; Brudzynski and Pniak, 2002; Knutson et al., 1998; Sales, 1972; Schwarting et al., 2007; White et al., 1990; Wöhr et al., 2008), serve a social and communicatory role: Presumably, rats emit 50-kHz calls to promote social contact, i.e. to signal their presence, and to maintain or reestablish social contacts; therefore, these calls might reflect a "desire for positive social engagement" (Blanchard et al.,1993; Brudzynski, 2005; Knutson et al., 1998; Panksepp and Burgdorf, 2000). Also, such calls are probably more likely when the animals are in a positive emotional state, which may be promoted by the external factor bedding.

However, other studies observed spontaneous 22-kHz calls when rats were isolated, but there the rats were either socially isolated over days and not only for a short period as in our case (Francis, 1977) or the details of housing/isolation were not specified (Anderson, 1954). Also, we extensively handle our rats before testing to reduce the aversiveness of such possibly confounding factors. We do not know whether similar handling was also applied in these older studies. Together, these methodological differences seem to account for the difference between our and the previous studies as they could affect the internal state of the animal and therefore its call behaviour.

4.2. Tickling

In the next two experiments, we used tickling to induce 50-kHz calls. This procedure, which simulates aspects of juvenile play behaviour, is performed manually by an experienced human experimenter; therefore, it was also termed "hetero-specific play". Several previous studies showed that tickling is very effective to induce 50-kHz calls (Burgdorf and Panksepp, 2001; Panksepp and Burgdorf, 2000; Schwarting et al., 2007). Compared to these studies, 50-kHz call rates in tickle test A were rather moderate. Also, a number of 22-kHz calls were emitted, indicating that the procedure also had some aversive effects. This outcome may be due to one or several of the following factors: age (i.e. the likelihood of emitting 50-kHz calls in response to being tickled seems to decline the more rats grow out of their play age), insufficient group size, insufficient tickling experience, or inadequate tickling components.

To rule out several of these factors, we performed an additional experiment (tickle test B), where we tested slightly larger groups, used more days of tickling experience, and applied a refined tickling protocol. Also, a cage test preceded the protocol (Schwarting et al., 2007) to rule out that subject-dependent differences in call rates flaw the subsequent tickling data. On the 5th day of this prolonged tickling protocol, we obtained substantial 50-kHz rates and no 22-kHz calls, indicating that the procedure was effective. This outcome was obtained despite testing adult subjects, indicating that 50-kHz calls in response to being tickled do not only occur in rats of the play age (see also Mällo et al., 2007; Schwarting et al., 2007). Besides 50-kHz calls, a few unusual low-frequency calls were observed which were of slightly higher ultrasonic frequency than 22-kHz calls, but much shorter than those. The nature of these rather rare calls is unclear and requires further experimental consideration in the future.

The major outcome of both experiments, however, is that rats tickled in cages with bedding emitted more 50-kHz calls than those tickled in bare ones. Thus, the factor bedding was not only effective in case of the cage test, where no additional manipulation is applied, but also when rats are manually tickled. Tickling, however, is dependent on the reciprocal interaction between a skilled experimenter and an individual rat. Therefore, this method is possibly flawed by methodological drawbacks, for example, performance of the experimenter or observer-expectancy effects (Solso and MacLin, 2001). Thus, despite taking various means to exclude such confounding variables, e.g. by applying standardized and improved tickling protocols we cannot completely exclude such impacts in case of tickling. Therefore, we abstained from further tests with this manipulation and switched to another, more easily controllable method to induce 50-kHz calls, namely systemic administration of the psychostimulant amphetamine, which has repeatedly been shown to elicit 50-kHz calls both, after systemic or intracerebral injections (Burgdorf et al., 2001a; Knutson et al., 1999; Thompson et al., 2006; Wintink and Brudzynski, 2001).

4.3. Activity box and amphetamine

Using an established repeated-measures design (Antoniou et al., 1998), we first habituated rats to the testing environment (no injection), then injected them with saline as a control, and finally with amphetamine itself. Initially, that is, when tested without drug or after saline injection, our rats emitted only few 50-kHz calls. In case of the 1st test (no injection) more calls were observed by rats tested on bedding, an outcome which qualitatively resembles that of the cage tests with bedding. Compared to the cage test, 50-kHz calls in the activity box were rather infrequent. This may be due to the fact, that the activity box is a novel environment unlike the home-like cage test. Thus, the unfamiliar activity box may have some anxiogenic properties, which are not outweighed by the familiar stimulus bedding. Therefore, 50-kHz calls, probably reflecting social approach motivation, were not or only weakly emitted since they require that the sender experiences a certain degree of safety.

When subsequently treated with an established dose of the psychomotor stimulant amphetamine (2.5 mg/kg (Wintink and Brudzynski, 2001)), many rats emitted 50-kHz vocalizations, and some of them at rather high rates. Importantly, these call rates were again more frequent in case of the bedded testing condition, indicating that this factor can also affect the outcome of a psychopharmacological manipulation. This substantial effect was only weakly paralleled by differences in visible behaviour, that is, the conventional measures of psychomotor activation. As expected, amphetamine led to enhanced locomotion, enhanced activity in the centre, and more rearing behaviour. Except for rearing time, which was higher in case of the bedded test condition, none of these measures yielded significant group differences between the bedded and non-bedded test condition. This effect was most pronounced around 21–25 min after injection. The time course of drug outcome on behavioural activity did not parallel that on ultrasonic vocalization, since the effects on vocalization peaked and started to decline again before those on psychomotor activation. These results indicate, for one, that 50-kHz calls are not a simple functional correlate of psychomotor activation (see also Burgdorf et al., 2001a), that is, they apparently do not reflect a motor artefact (Blumberg, 1992). Secondly, since peak vocalization preceded psychomotor activation, the two effects seem to be mediated by neuronal mechanisms, which are at least partly distinct.

It is known that amphetamine works mainly by enhancing the efficacy of endogenous biogenic amines, like dopamine or noradrenaline. Interestingly, the peak effect of amphetamine on 50-kHz calls was observed rather early after injection, which may correspond to the boost phase of extracellular dopamine in the brain, as measured with in-vivo microdialysis after sc amphetamine (Kuczenski et al., 1995). Even more, work in humans has shown that such psychostimulant-induced dopaminergic boost phases parallel and probably even determine the subjective experience of 'high' (Volkow et al., 2004). Therefore, we suggest that the 50-kHz calls reflected a positive emotional state, which was more pronounced when amphetamine was experienced in an environment containing bedding.

4.4. The role of bedding

These experiments raise the functional question why bedding had such strong effects on the occurrence of ultrasonic vocalization, as

seen with tickling, in the cage test, or under amphetamine. The calls investigated here are usually interpreted in terms of emotion, motivation and communication. Thus, it has been suggested that they index a positive emotional state, especially one of appetitiveness or wanting (Knutson et al., 2002), which in the case of tickling or the cage test can serve as a social signal for approach (Wöhr and Schwarting, 2007), like "come on and play". Emotional science has repeatedly shown that the likelihood of a positive social signal (like laughing or smiling in humans) is higher, when the signalling subject is in a positive emotional state. One efficient condition for such a state is familiarity (Titchener, 1910), that is, the bedding, on which the rats were tested, is a typical feature of their life-long housing environment. Therefore, bedding can index familiarity and also perhaps safety, which is emotionally positive.

4.5. Choice test

To further test this hypothesis, we performed a final experiment where we applied a choice procedure by means of a conventional place preference apparatus with bedded and non-bedded compartments. As expected, rats spent more time in the bedded compartment, that is, they showed a preference for this environment. Moreover, they emitted some 50-kHz calls in the apparatus, which were more frequent in the bedded compartment both, in absolute terms and when expressed per time spent. These vocalizations are probably not due to novelty, a) since previous work showed that novel stimuli are more likely to suppress rather than induce 50-kHz calls (Knutson et al., 2002), and b) since calls were more frequent in that part of the testing environment, which contained a familiar component, namely bedding. This component probably signals security. Therefore, we assume that the bedding provided a positive and appetitive stimulus. This stimulus resulted in a positive subjective state, which, in turn, increased the likelihood that the animal emitted 50-kHz calls.

4.6. Conclusions and general remarks

We think that the present findings are of potential importance for several scientific issues: For one, our data show that rather subtle environmental modifications, here bedding (for another example see Thiel et al., 2000), can have substantial effects on rats, and these effects can become detectable given the adequate measure. So far, research on bedding has focused on its role for housing and animal welfare (McMillan, 2005), that is, rodents are known to prefer bedded housing conditions and may grow up healthier in them (Satinder, 1967; Sorensen et al., 2004). Besides, bedding has repeatedly been used as an experimental stimulus, that is, different kinds of soiled beddings were presented to rats to signal own and different social stimuli, especially with respect to sexuality (Bressler and Baum, 1996). Also, bedding is used in tests of anxiety, like marble burying or shock probe tests (Terlecki et al., 1979), where rodents use bedding to occlude aversive stimuli. The present work adds a new feature to the behavioural research field, since it shows that the simple presence of unsoiled bedding can apparently modify the affective quality of a test situation. Usually, behavioural tests (like open-fields) are run without any bedding, but researchers should consider to add bedding to it, at least if they want to reduce the aversiveness of such test conditions.

Also, our work shows that 50-kHz vocalizations seem to reveal mechanisms, which are not necessarily gauged by the prevailing measures of visible behaviour. This can be of importance for a number of studies, especially behavioural ones on emotion and motivation, where conventional measures (like behavioural activity, approach, consumption, place preference etc.) should be escorted more often by refined measures of ultrasonic vocalization (Knutson et al., 1999). Apart from that, ultrasonic vocalization should be considered as a supplement when studying drugs of abuse (Burgdorf et al., 2007), especially psychostimulants, or even direct brain manipulations, like lesion. Thereby, functional differences may become detectable between treatments, or even between subjects within a given treatment, which expand or even modify the interpretation provided by overt (i.e. visible) behavioural measures. Besides these psychological issues, differences in vocalization must be paralleled by differences in the brain. For example, in case of our amphetamine study one could expect partly distinct patterns of brain activation under bedded vs. unbedded conditions, which can be studied with neuronal methods like marking the activation of immediate early genes. These neural patterns should not only encompass the sensory input side (like tactile and olfactory), but also and most importantly, brain areas relevant for affective processing.

Finally, 50-kHz calls may provide a link to the subject's positive emotional state, not in terms of a simple copy or readout, but as a communicational sign, which is closely related and largely dependent on affect, in general, or even feeling, in detail. In that respect, such calls share similarities to human facial and vocal expressions, which work via a communicative route. Such signs do not simply mirror an affective state, but they can be strongly determined by it. Nowadays, many scientists seriously consider the avenue that feelings, at least in a very basic and nonverbal sense, do also work in rodents (Cabanac et al., 2009; Panksepp, 1998). Clearly, more and more refined experiments will be necessary to further study this theoretical issue, and here rodent ultrasonic vocalization may serve as a useful probe.

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