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Ultrasonic communication in rats: Effects of morphine and naloxone on vocal and behavioral responses to playback of 50-kHz vocalizations

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ABSTRACT

Rats emit ultrasonic vocalizations and it was hypothesized that these vocalizations have an important role in intra-specific communication. Recently, we demonstrated that playback of 50-kHz ultrasonic vocalizations can induce social approach, indicating that 50-kHz calls can serve to (re)establish or to maintain social contact. It is known that endogenous opioids are implicated in the regulation of social behavior, particularly in rough and tumble play. Here, we tested whether administration of opioid ligands can affect social approach in response to playback of 50-kHz calls in juvenile and adult rats. Rats were either treated with 1 mg/kg naloxone, 1 mg/kg morphine, or with saline vehicle. Administration of opioid ligands affected social approach at both ages. Specifically, in juvenile and adult rats, social approach displayed in response to playback of 50-kHz calls was reduced in case of naloxone treatment, but enhanced with morphine. Furthermore, juvenile rats treated with saline or morphine emitted a substantial amount of ultrasonic vocalizations in response to the playback of 50-kHz calls. Such ultrasonic calling was not seen in naloxone treated rats. Importantly, these drug-dependent differences were stimulus-specific, i.e. seen only in response to playback of 50-kHz calls and not in response to playback of background noise. The present finding that opioid ligands can affect social approach and ultrasonic vocalizations induced by playback of 50-kHz calls, indicates that an important feature of social interaction in rats, namely ultrasonic communication, is at least partially regulated by endogenous opioids.

1. Introduction

Endogenous opioids have been implicated in the neurochemical control of various types of social behavior in many species (Panksepp et al., 1980, 1997). Therefore, it was hypothesized that the endogenous opioid system might play a role in psychiatric disorders characterized by deficits in social behavior such as autism (Moles et al., 2004; Sahley and Panksepp, 1987).

In the rat, it is known that the endogenous opioid system is involved in the isolation-induced ultrasonic vocalization response of pups (Carden and Hofer, 1990a,b; Kehoe and Blass, 1986), maternal behavior (Bridges and Grimm, 1982), rough and tumble play (Panksepp et al., 1984; Vanderschuren et al., 1997), and sexual behavior (Gessa et al., 1979). Among them, effects of exogenous opiates were extensively studied in the context of rough and tumble play in juvenile rats. These studies consistently showed that the μ-opioid-receptor-agonist morphine (MOR) increases rough and tumble play, as well as other μ-opioid-receptor-agonists such as methadone, fentanyl or beta-endorphin (Niesink and Van Ree, 1989; Normansell and Panksepp, 1990; Panksepp et al., 1985; Siviy and Panksepp, 1985; Vanderschuren et al., 1995a,b, 1996, 1997), while a decrease in rough and tumble play was observed after administration of μ-opioid-receptor-antagonists such as naloxone (NAL), naltrexone or beta-funaltrexamine (Beatty and Costello, 1982; Jalowiec et al., 1989; Niesink and Van Ree, 1989; Normansell and Panksepp, 1990; Panksepp et al., 1985; Siegel and Jensen, 1985, 1986; Siviy and Panksepp, 1985; Vanderschuren et al., 1995b). Conversely to the μ-opioid-receptor, δ- and κ-receptors seem not to be specifically involved in the regulation of rough and tumble play (Vanderschuren et al., 1995b).

In contrast to the clear effects of exogenous opiates on rough and tumble play, conflicting results were obtained in other social contexts. On the one side, it was shown that NAL reduces social interaction (File, 1980) and social locomotor activity (DeRossett and Holtzman, 1982; Dokla, 1992; File, 1980). On the other side, however, Panksepp et al. (1979) demonstrated that MOR reduces social cohesion, i.e. the time spent in proximity with other rats, as well as social locomotor activity. Also, Panksepp et al. (1980) anecdotally reported that NAL increases the preference for a social reward while MOR enhances the preference for a food reward. Furthermore, MOR reduced social investigation in a recently developed social-hole-task (Deak et al., 2009), and another μ-opioid-receptor-agonist, methadone, was shown to lower levels of social locomotor activity, time spent in social contact, and grooming each other (Plonsky and Freeman, 1982).

Since social behavior is highly complex, it is obvious that these conflicting results might be attributable to various factors. One way to address this problem is to reduce the enormous level of complexity by

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studying important features of social behaviors selectively. Among these important features are ultrasonic vocalizations. Juvenile and adult rats emit two distinct types of ultrasonic vocalizations depending on their affective state. Rats emit 22-kHz calls in aversive situations such as social defeat (Burgdorf et al., 2008a; Frank et al., 2006; Sales, 1972a; Thomas et al., 1983), predator exposure (Blanchard et al., 1990, 1991, 1992; Shepherd et al., 1992), delivery of electric shock (Borta et al., 2006; Choi and Brown, 2003; Molewijk et al., 1995; Van der Poel et al., 1989; Wöhr and Schwarting, 2008a,b; Wöhr et al., 2005), withdrawl from opiates, or psychostimulants when prompted by a mild startling stimulus (Barros and Miczek, 1996; Covington and Miczek, 2003; Mutschler and Miczek, 1998; Vivian and Miczek, 1991), and unexpected termination of rewarding stimuli (Burgdorf et al., 2000). In appetitive situations, on the other hand, rats emit 50-kHz calls. They are elicited most robustly during social interactions, in particular rough and tumble play or tickling in juvenile rats (Brunelli et al., 2006; Burgdorf et al., 2008a; Knutson et al., 1998; Panksepp and Burgdorf, 2000, 2003; Schwarting et al., 2007) and during mating in adult rats (Burgdorf et al., 2008a; Sales, 1972b; McIntosh et al., 1978).

Recently, the idea came up that 50-kHz calls serve as contact calls to (re)establish or maintain contact with conspecifics. Firstly, this view is indicated by studies on the effect of social context on call production in the sender. Brudzynski and Pniak (2002) found that rats emit 50-kHz calls in a dose-dependent manner when exposed to odor of conspecifics, indicating that the production of 50-kHz calls is driven by potential social contact. Also, 50-kHz calls were detected after separation of conspecifics from each other during short social isolation (Schwarting et al., 2007; Wöhr et al., 2008). Remarkably, the propensity to call differed dependent on the time-point of the last social contact, i.e. rats emitted 50-kHz calls primarily initially after separation from their cage mate (Wöhr et al., 2008). Furthermore, we found that not only the animal, which was isolated in a new housing cage, emitted 50-kHz calls, but also its cage mate that remained alone in the home cage after the removal of the test rat (Wöhr et al., 2008). Secondly, a communicative function of 50-kHz calls is further provided by studies on the effects of 50-kHz calls on behavior of the receiver. Panksepp et al. (2002) observed that rats spent more time with conspecifics, which vocalize a lot, than with ones, which display less calling behavior. This is in line with findings showing that deafening or devocalizing rats changes rough and tumble play (Siviy and Panksepp, 1987). Finally, by performing a playback study, we clearly demonstrated that 50-kHz calls can induce social approach in the receiver (Wöhr and Schwarting, 2007). This behavioral response was specific to 50-kHz calls, since it was not observed in response to 22-kHz calls, which induce locomotor inhibition (Brudzynski and Chiu, 1995; Burman et al., 2007; Sales, 1991; Wöhr and Schwarting, 2007) and activation of brain areas implicated in anxiety and fear (Sadananda et al., 2008). Conversely, social approach in response to 50-kHz calls was paralleled by an activation of brain areas implicated in reward processing such as the nucleus accumbens (Sadananda et al., 2008), an area where opioids exert their effects on social behavior (Panksepp and Bishop, 1981; Vanderschuren et al., 1995c).

Therefore, we tested here whether the administration of opioid ligands can affect social approach displayed in response to playback of 50-kHz calls in juvenile and adult rats.

2. Materials and methods

2.1. Animals and housing

In total, 48 naïve male Wistar rats (HsdCpb:WU, Harlan-Winkelmann, Borchen, Germany) served as subjects. Among them were 24 juvenile rats, weighing 73.21 ± 1.82 g (range: 60.00–93.50 g; about 4 weeks of age) on the test day, and 24 adult rats, weighing 314.98 ± 2.64 g (range: 297.00–343.00 g; about 12 weeks of age) on the test day. Animals were housed in groups of 6 on Tapvei peeled aspen bedding (indulab ag, Gams, Switzerland) in polycarbonate Macrolon type IV cages (size: 380 × 200 × 590 mm, plus high stainless steel covers) in an animal room with a 12:12 h light/dark cycle (lights on 7–19 h) where the environmental temperature was maintained between 21 and 24 °C (humidity: 30–40%). Lab chow (Altromin, Lage, Germany) and water (0.0004% HCl-solution) were available ad libitum. After delivery, all animals were allowed to adjust to the housing and light conditions for about 1 week and were handled for 3 days in a standardized way (5 min each day) prior testing.

2.2. Experimental setting

Testing was performed on an elevated radial maze (for details see: Wöhr and Schwarting, 2007). Acoustic stimuli were presented through an ultrasonic speaker (ScanSpeak, Avisoft Bioacoustics, Berlin, Germany) using an external sound card with a sampling rate of 192 kHz (Fire Wire Audio Capture FA-101, Edirol, London, UK; for details see: Wöhr and Schwarting, 2007) The loudspeaker was placed 20 cm away from the end of one arm at a height of 52 cm above the floor. Testing was performed under red light (~10 lux) in a testing room with no other rats present. All behavioral tests were conducted between 9 and 17 h. Prior to each test, behavioral equipment was cleaned using 0.1% acetic acid solution followed by drying.

2.3. Acoustic stimuli

Natural 50-kHz calls and background noise served as the acoustic stimuli (for a detailed description of the stimulus material and exemplary spectrograms see: Wöhr and Schwarting, 2007). Playback of 50-kHz calls consisted of 221 natural 50-kHz calls recorded from an adult male Wistar rat during exploration of a cage containing scents from a cage mate (for setting and recording see: Wöhr et al., 2008). This context was chosen for recording of 50-kHz calls, since playback of 50-kHz calls recorded during tickling or exploration of an empty clean cage had no or only minor effects on the behavior of the recipient (Burman et al., 2007; Endres et al., 2007). Playback of background noise consisted of background noise recorded from a rat exploring an area with bedding (for setting and recording see: Wöhr et al., 2008). Background noise was presented to control for unspecific effects due to the background noise normally present during the playback of 50-kHz calls. Both stimuli, i.e. natural 50-kHz calls with background noise and background noise alone, were presented for 1 min with a sampling rate of 192 kHz in 16 bit format.

2.4. Experimental procedure

A given animal was placed onto the central platform of the radial maze, facing the arm opposite to the loudspeaker. After an initial habituation phase of 15 min where no acoustic stimuli were presented, the rat was exposed to two phases of acoustic stimulation for 1 min, each followed by an inter-stimulus-interval of 10 min. Each rat was exposed to playback of 50-kHz calls and background noise. Stimuli were presented in a counter-balanced manner to account for the possible impact of serial effects.

2.5. Independent variable

Juvenile or adult rats were either treated intraperitoneally with the μ-opioid-receptor-antagonist naloxone (1.0 mg/kg; NAL), the μ-opioid-receptor-agonist morphine (1.0 mg/kg; MOR), or isotonic 0.9% NaCl solution (saline; SAL) 5 min before testing (volume: 0.5 mg/ml in juveniles; 1.0 mg/ml in adults). Doses were chosen based on studies on rough and tumble play, where these doses are commonly used, since they exert strong effects on social behavior without affecting general locomotor activity (Beatty and Costello, 1982; Niesink and Van Ree, 1989; Normansell and Panksepp, 1990; Panksepp et al., 1985; Siegel and Jensen, 1985, 1986; Siviy and Panksepp, 1985; Vanderschuren et al., 1995a, 1996). Naloxone hydrochloride dehydrate and morphine sulfate salt pentahydrate were purchased from Sigma-Aldrich Chemie GmbH

(Taufkirchen, Germany). Drugs were dissolved in isotonic 0.9% NaCl solution. Solutions were freshly prepared in plastic containers on the day of the experiment. Animals were randomly assigned to drug-treatment and each animal was used only once.

2.6. Dependent variables

2.6.1. Recording and analysis of animal activity

Behavior was monitored by a video camera (Panasonic WV-BP 330/GE, Hamburg, Germany) from about 150 cm above the maze, which fed into DVD recorder (DVR-3100 S, Pioneer, Willich, Germany). Behavioral analysis was performed using an automated video tracking system (Ethovision, Noldus, Wageningen, The Netherlands). The following three parameters were determined: 1) total distance travelled (cm), 2) the number of arm entries into the three arms proximal to or distal from the ultrasonic loudspeaker, and 3) the time spent therein. For the automated analysis, input filters were activated to avoid an over-estimation of locomotor activity due to head-movements. A minimal distance moved of 8 cm was used for the total distance travelled, whereas a minimal distance moved of 3 cm was used for arm entries and the time spent therein.

2.6.2. Recording and analysis of ultrasonic vocalization

Playback of acoustic stimuli and potential ultrasonic calls uttered by the test subject itself were monitored by two UltraSoundGate Condenser Microphones (CM 16; Avisoft Bioacoustics) placed 20 cm away from the maze at a height of 55 cm above the floor (for details see: Wöhr and Schwarting, 2007). Acoustic data were recorded with a sampling rate of 214,285 Hz in16 bit format by Avisoft RECORDER (version 2.7; Avisoft Bioacoustics). For acoustical analysis, recordings were transferred to SASLab Pro (version 4.38; Avisoft Bioacoustics) and a fast Fourier transform was conducted (512 FFT-length, 100% frame, Hamming window and 75% time window overlap), resulting in high resolution spectrograms (frequency resolution: 488 Hz; time resolution: 0.512 ms). An experienced user counted the number of ultrasonic vocalizations.

2.7. Statistical analysis

An ANOVA for repeated measurements with the within-subject factor test phase (before, during and after playback) and two between-subject factors, namely age (juvenile and adult) and drug-treatment (NAL, SAL and MOR), was calculated to test whether locomotor activity and ultrasonic calling are affected by presentation of acoustic stimuli, and whether a potential effect is modulated by age or drug-treatment. An ANOVA for repeated measurements with the within-subject factor preference (proximal and distal) and two between-subject factors, namely age (juvenile and adult) and drug-treatment (NAL, SAL and MOR), was calculated to test whether stimulus-directed locomotor activity is affected by presentation of acoustic stimuli, and whether a potential effect is modulated by age or drug-treatment. The ANOVA was followed by post-hoc tests (LSD) or paired *t*-tests when appropriate ($p<0.050$). A *p*-value of <0.050 was considered statistically significant.

3. Results

3.1. Locomotor activity (Fig. 1)

3.1.1. Playback of 50-kHz ultrasonic vocalizations

Juvenile and adult rats displayed increased locomotor activity in response to playback of 50-kHz calls (main effect test phase: $F_{2,84}=23.855$; $p<0.001$). Rats increased their distance traveled from 59.32 ± 7.49 cm/min in the minutes before stimulus application to 129.96 ± 13.45 cm/min during playback of 50-kHz calls ($t_{47}=-5.233$, $p<0.001$), followed by a reduction after cessation of playback to 65.42 ± 6.69 cm/min ($t_{47}=4.850$, $p<0.001$), while there was no difference in locomotor activity before versus after playback of 50-kHz calls ($t_{47}=-0.738$, $p=0.464$).

The increase in locomotor activity during playback of 50-kHz calls was more pronounced in juvenile than in adult rats (interaction test phase × age: $F_{2,84}=6.261$; $p=0.005$). Specifically, juvenile rats increased their distance traveled from 55.28 ± 11.30 cm/min in the minutes before stimulus application to 163.28 ± 20.43 cm/min during playback of 50-kHz calls ($t_{23}=-5.575$, $p<0.001$), followed by a reduction after cessation of playback to 67.43 ± 7.74 cm/min ($t_{23}=4.975$, $p<0.001$). Adult rats, however, increased their distance traveled only slightly from 63.36 ± 10.02 cm/min in the minutes before stimulus application to 96.64 ± 15.02 cm/min during playback of 50-kHz calls ($t_{23}=-2.113$, $p=0.046$), followed by a slight reduction after cessation of playback to 63.41 ± 11.08 cm/min ($t_{23}=2.032$, $p=0.054$). Locomotor activity before versus after playback of 50-kHz calls did not differ in juvenile ($t_{23}=-1.164$, $p=0.256$) and adult rats ($t_{23}=-0.003$, $p=0.997$).

The increase in locomotor activity during playback of 50-kHz calls was not dependent on drug-treatment (interaction test phase × drug-treatment: $F_{4,84}=0.736$; $p=0.548$; interaction test phase × age × drug-treatment: $F_{2,84}=1.203$; $p=0.316$).

However, there was an overall effect of drug-treatment (main effect drug-treatment: $F_{2,42}=9.196$; $p<0.001$), but not of age (main effect age: $F_{1,42}=3.198$; $p=0.081$; interaction age × drug-treatment: $F_{2,42}=0.309$; $p=0.736$). Irrespective of test phase, locomotor activity was higher in MOR treated rats (115.30 ± 8.68 cm/min) than in SAL treated rats (85.37 ± 8.68 cm/min), which, in turn, was higher than in NAL treated rats (54.04 ± 8.68 cm/min; post-hoc tests: all *p*-values <0.050).

3.1.2. Playback of background noise

Juvenile and adult rats displayed decreased locomotor activity in response to playback of background noise (main effect test phase: $F_{2,84}=12.801$; $p<0.001$). Rats decreased their distance traveled from 61.46 ± 6.50 cm/min in the minutes before stimulus application to 33.08 ± 7.80 cm/min during playback of background noise ($t_{47}=3.889$, $p<0.001$). The reduction was still evident after cessation of playback, where rats traveled 30.31 ± 5.07 cm/min, which was similar to the distance traveled during playback ($t_{47}=0.391$, $p<0.697$), but lower in comparison to the distance traveled before playback ($t_{47}=5.515$, $p<0.001$).

The decrease in locomotor activity during playback of background noise was not dependent on age (interaction test phase × age: $F_{2,84}=2.228$; $p=0.120$) and drug-treatment (interaction test phase × drug-treatment: $F_{4,84}=0.369$; $p=0.811$; interaction test phase × age × drug-treatment: $F_{2,84}=0.366$; $p=0.813$).

Also, there was no overall effect of age (main effect age: $F_{1,42}=0.916$; $p=0.344$) and drug-treatment (main effect drug-treatment: $F_{2,42}=2.045$; $p=0.142$; interaction age × drug-treatment: $F_{2,42}=0.305$; $p=0.739$).

3.1.3. Summary

Playback of 50-kHz calls and playback of background noise exerted opposite effects on locomotor activity. While playback of 50-kHz calls increased locomotor activity, playback of background noise decreased it. This shows that the increase in locomotor activity was specific to 50-kHz calls. Furthermore, the locomotor activation in response to 50-kHz calls was age-dependent. Playback of 50-kHz calls led to a stronger increase in locomotor activity in juvenile than in adult rats. However, the strength of locomotor activation seen in response to playback of 50-kHz calls was independent from drug-treatment.

3.2. Stimulus-directed locomotor activity (Figs. 2 and 3)

3.2.1. Playback of 50-kHz ultrasonic vocalizations—arm entries

The increase in locomotor activity in response to playback of 50-kHz calls was directed towards the stimulus source. This is indicated by a comparison between the numbers of entries (n/min) into the proximal arms with those into the distal arms (main effect preference:

$F_{1,42} = 90.452$; $p < 0.001$). During playback of 50-kHz calls, rats entered the proximal arms 2.10 ± 0.21 n/min, while the distal ones only 0.25 ± 0.07 n/min.

The preference for proximal arms during playback of 50-kHz calls was more pronounced in juvenile than in adult rats (interaction preference × age: $F_{1,42} = 8.325$; $p = 0.006$). Specifically, juvenile rats entered the proximal arms 2.63 ± 0.29 n/min, while the distal arms only 0.21 ± 0.08 n/min ($t_{23} = -6.928$; $p < 0.001$). Adult rats, however, entered the proximal arms 1.58 ± 0.28 n/min, while the distal arms only 0.30 ± 0.11 n/min ($t_{23} = -4.403$; $p < 0.001$).

The preference for proximal arms during playback of 50-kHz calls was also dependent on drug-treatment (interaction preference × drug-treatment: $F_{2,42} = 5.835$; $p = 0.006$; interaction preference × age × drug-treatment: $F_{2,42} = 0.240$; $p = 0.788$). The interaction between preference and drug-treatment was due to drug-dependent differences in the time spent on proximal arms ($F_{2,48} = 8.406$; $p = 0.001$), since the time spent on distal arms was unchanged by drug-administration ($F_{2,48} = 0.244$; $p = 0.784$). NAL treated rats entered the proximal arms less often than SAL treated rats (post-hoc test: $p = 0.024$) or MOR treated rats (post-hoc test: $p < 0.001$), while no difference was observed between SAL and MOR treated rats (post-hoc test: $p = 0.087$; for significance levels for juvenile and adult rats separately see Fig. 2).

In juvenile rats, when comparing the entries into proximal arms with those into distal arms during playback of 50-kHz calls, only a moderate preference was observed after treatment with NAL. Rats treated with NAL entered the proximal arms 1.50 ± 0.33 n/min while only 0.13 ± 0.13 n/min the distal ones ($t_7 = 4.245$, $p = 0.004$). SAL treated rats, however, entered the proximal arms 2.88 ± 0.52 n/min while only 0.25 ± 0.16 n/min the distal ones ($t_7 = 4.406$, $p = 0.003$). Finally, the clearest preference towards the stimulus source was displayed by MOR treated rats. They entered the proximal arms 3.50 ± 0.38 n/min while only 0.25 ± 0.16 n/min the distal ones ($t_7 = 10.370$, $p < 0.001$). Despite the treatment-induced modulation of the number of entries into the proximal arms during playback of 50-kHz calls, more proximal arm entries were observed in response to 50-kHz calls irrespective of drug-treatment when comparing entries into the proximal arms in response to 50-kHz calls and background noise (NAL: $t_7 = -3.862$, $p = 0.006$; SAL: $t_7 = -5.185$, $p = 0.001$, MOR: $t_7 = -5.227$, $p = 0.001$).

In adult rats, no preference was observed in NAL treated rat, which entered the proximal arms 0.88 ± 0.23 n/min while 0.25 ± 0.25 n/min the distal ones ($t_7 = 1.488$, $p = 0.180$). However, a preference towards proximal arms was observed in SAL treated rats, which entered the proximal arms 1.50 ± 0.42 n/min while only 0.25 ± 0.16 n/min the distal ones ($t_7 = 2.546$, $p = 0.038$). Finally, a clear preference towards the stimulus source was displayed by MOR treated rats. They entered the proximal arms 2.38 ± 0.60 n/min while only 0.38 ± 0.18 n/min the distal ones ($t_7 = 3.191$, $p = 0.015$). Despite the treatment-induced modulation of the number of entries into the proximal arms during playback of 50-kHz calls, more proximal arm entries were observed in response to 50-kHz calls irrespective of drug-treatment when comparing entries into the proximal arms in response to 50-kHz calls and background noise (NAL: $t_7 = -3.000$, $p = 0.020$; SAL: $t_7 = -2.646$, $p = 0.033$; MOR: $t_7 = -2.935$, $p = 0.022$).

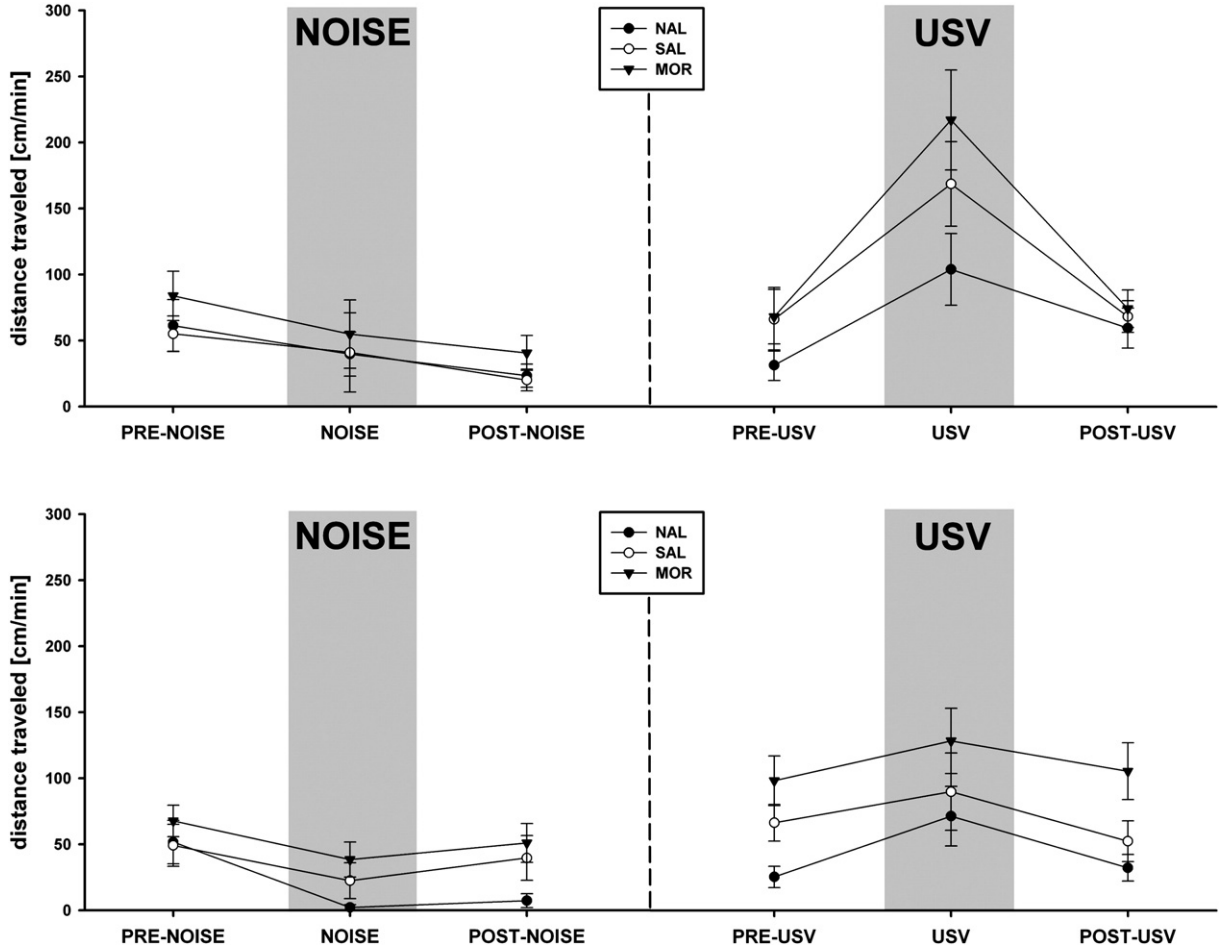

Fig. 1. Locomotor activity of juvenile (upper panel) and adult (lower panel) rats treated with NAL (naloxone; black circles), SAL (saline; white circles) or MOR (morphine; black triangles). The left graphs depict the distance travelled (cm/min) for test phases before (PRE-NOISE), during (NOISE), and after (POST-NOISE) presentation of background noise. The right graphs depict these measures for test phases before (PRE-USV), during (USV), and after (POST-USV) presentation of 50-kHz ultrasonic vocalizations. The values before or after stimulus presentation reflect the averaged values measured during the respective five minute periods. Values reflect means ± SEM per minute.

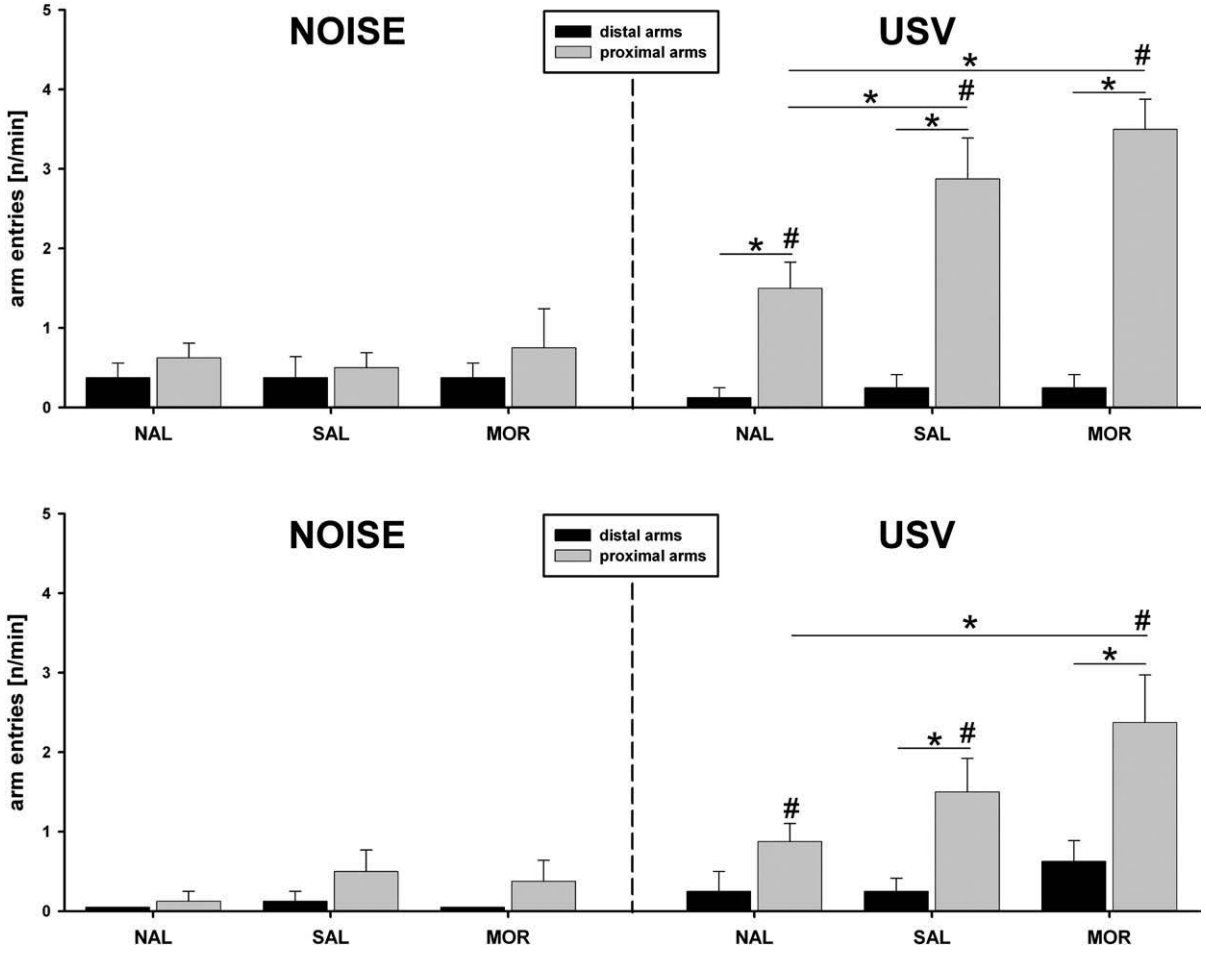

Fig. 2. Stimulus-directed locomotor activity of juvenile (upper panel) and adult (lower panel) rats treated with NAL (naloxone), SAL (saline) or MOR (morphine). The number of entries (n/min) into the distal (black bars) or proximal (grey bars) arms from the loudspeaker is given for presentation of background noise (NOISE, left graphs) and 50-kHz ultrasonic vocalizations (USV, right graphs). Values reflect means ± SEM per minute. Comparisons between entries into distal or proximal arms with $p<0.050$ are marked with asterisks: *.Comparisons between entries into proximal arms during playback of background noise and 50-kHz calls with $p<0.050$ are marked with a pound sign: #.

Also, there was an overall effect of age (main effect age: $F_{1,42}=6.794$; $p=0.013$) and drug-treatment (main effect drug-treatment: $F_{2,42}=8.721$; $p=0.001$; interaction age × drug-treatment: 0.475; $p=0.625$). Juvenile rats entered proximal and distal arms more often (1.42 ± 0.13) than adult rats (0.94 ± 0.13). Furthermore, NAL treated rats (0.69 ± 0.16) entered proximal and distal arms less often than SAL (1.22 ± 0.16; post-hoc test: $p=0.023$) or MOR treated rats (1.63 ± 0.16; post-hoc test: $p<0.001$), while the latter did not differ (post-hoc test: $p=0.078$).

3.2.2. Playback of 50-kHz ultrasonic vocalizations—time spent on arms

In line with a higher number of entries into the proximal arms than into the distal arms during playback of 50-kHz calls, rats spent more time in the proximal arms than in the distal arms (main effect preference: $F_{1,42}=90.787$; $p<0.001$). During playback of 50-kHz calls, rats spent 27.27 ± 2.63 s/min on the proximal arms, while only 1.84 ± 0.78 s/min on the distal ones.

Also in accordance to the number of arm entries, the preference for proximal arms during playback of 50-kHz calls was more pronounced in juvenile than in adult rats (interaction preference × age: $F_{1,42}=7.119$; $p=0.011$). Specifically, juvenile rats spent 35.02 ± 3.59 s/min on proximal arms, while only 2.47 ± 1.35 s/min on distal ones ($t_{23}=7.768$; $p<0.001$). Adult rats, however, spent 19.52 ± 3.20 s/min on proximal arms, while only 1.21 ± 0.80 s/min on distal ones ($t_{23}=5.226$; $p<0.001$).

The preference for proximal arms during playback of 50-kHz calls was not dependent on drug-treatment (interaction preference × drug-treatment: $F_{2,42}=3.019$; $p=0.060$; interaction preference × age × drug-treatment: $F_{2,42}=0.064$; $p=0.938$). Nevertheless, there were drug-dependent differences in the time spent proximal ($F_{2,48}=3.419$; $p=0.042$), while the time spent distal was unchanged by drug-administration ($F_{2,48}=0.434$; $p=0.651$). NAL treated rats entered the proximal arms less often than SAL treated rats (post-hoc test: $p=0.020$) or MOR treated rats (post-hoc test: $p=0.045$), while no difference was observed between SAL and MOR treated rats (post-hoc test: $p=0.730$; for significance levels for juvenile and adult rats separately see Fig. 3).

In juvenile rats, when comparing the time spent in proximal arms with that spent in distal arms during playback of 50-kHz calls, only a moderate preference was observed after treatment with NAL. Rats treated with NAL spent 26.63 ± 7.69 s/min in the proximal arms during playback of 50-kHz calls while only 2.58 ± 2.04 s/min in distal ones ($t_7=2.822$, $p=0.026$). SAL treated rats spent 41.78 ± 3.54 s/min in the proximal arms while only 3.68 ± 3.51 s/min in distal ones ($t_7=6.164$, $p<0.001$), and MOR treated rats spent 36.65 ± 6.18 s/min in the proximal arms while only 1.15 ± 0.96 s/min in distal ones ($t_7=5.217$, $p=0.001$). Furthermore, when comparing the time spent on proximal arms in response to 50-kHz calls and background noise, higher values were observed in response to 50-kHz calls in MOR treated rats ($t_7=-3.627$, $p=0.008$), but not in NAL treated rats ($t_7=-0.940$, $p=0.379$) or SAL treated rats ($t_7=-1.205$, $p=0.267$).

In adult rats, no preference for the stimulus source was observed in NAL treated rats, but in SAL or MOR treated rats. NAL treated rats spent 10.88 ± 4.59 s/min in the proximal arms during playback of 50-kHz calls

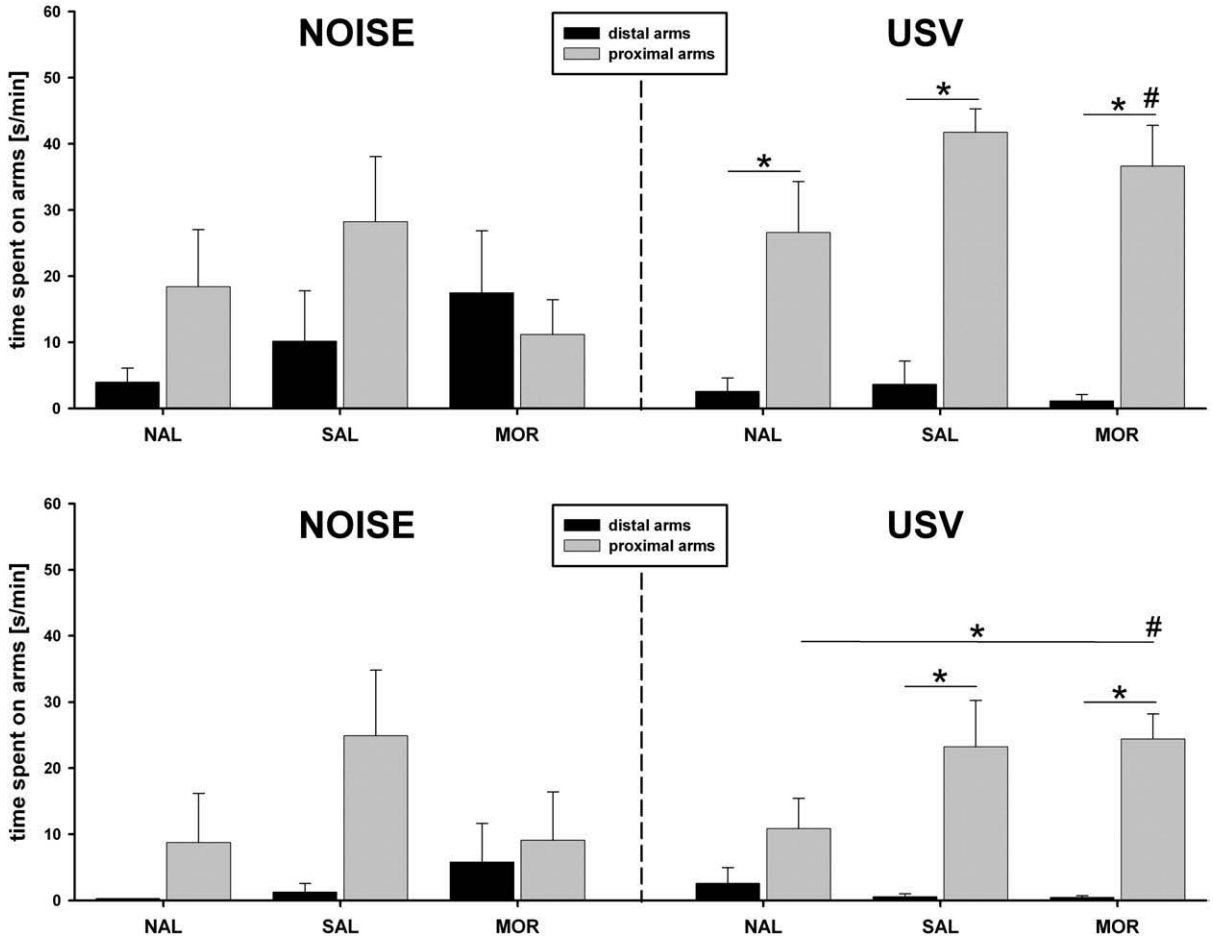

Fig. 3. Stimulus-directed locomotor activity of juvenile (upper panel) and adult (lower panel) rats treated with NAL (naloxone), SAL (saline) or MOR (morphine). The time spent (s/min) in the distal (black bars) or proximal (grey bars) arms from the loudspeaker is given for presentation of background noise (NOISE, left graphs) and 50-kHz ultrasonic vocalizations (USV, right graphs). Values reflect means ± SEM per minute. Comparisons between time spent in distal or proximal arms with $p < 0.050$ are marked with asterisks: *. Comparisons between time spent in proximal arms during playback of background noise and 50-kHz calls with $p < 0.050$ are marked with a pound sign: #.

while 2.60 ± 2.38 s/min in distal ones ($t_7 = 1.435$, $p = 0.195$). SAL treated rats spent 23.25 ± 7.02 s/min in the proximal arms while only 0.55 ± 0.50 s/min in distal ones ($t_7 = 3.125$, $p = 0.017$) and MOR treated rats spent 24.43 ± 3.78 s/min in the proximal arms while only 0.48 ± 0.24 s/min in distal ones ($t_7 = 6.578$, $p < 0.001$). Furthermore, when comparing the time spent on proximal arms in response to 50-kHz calls and background noise, higher values were observed in response to 50-kHz calls in MOR treated rats ($t_7 = -3.200$, $p = 0.015$), but not in NAL treated rats ($t_7 = -0.226$, $p = 0.827$) or SAL treated rats ($t_7 = 0.152$, $p = 0.883$).

Also, there was an overall effect of age (main effect age: $F_{1,42} = 14.124$; $p = 0.001$) and drug-treatment (main effect drug-treatment: $F_{2,42} = 3.213$; $p = 0.050$; interaction age × drug-treatment: $F_{2,42} = 0.334$; $p = 0.718$). Juvenile rats spent more time on proximal and distal arms (18.74 ± 1.58 s/min) than adult rats (10.36 ± 1.58 s/min). Furthermore, NAL treated rats spent less time on proximal and distal arms (10.67 ± 1.93 s/min) than SAL treated rats (17.31 ± 1.93 s/min; post-hoc test: $p = 0.019$), while SAL and MOR treated rats (15.68 ± 1.93 s/min; post-hoc test: $p = 0.552$) as well as NAL and MOR treated rats did not differ (post-hoc test: $p = 0.074$).

3.2.3. Playback of background noise—arm entries

A weak preference for the proximal arms was observed in response to playback of background noise. This is indicated by a comparison between the numbers of entries into the proximal arms with those into the distal arms (main effect preference: $F_{1,42} = 5.502$; $p = 0.024$). During playback of background noise, rats entered the proximal arms 0.48 ± 0.11 n/min, while the distal ones only 0.21 ± 0.07 n/min. It is important to note, however, that the preference towards the stimulus source in response to playback of background noise was smaller than that observed in response to playback of 50-kHz calls ($t_{47} = -6.604$; $p < 0.001$). Even more importantly, the preference for proximal arms during playback of background noise was not dependent on age (interaction preference × age: $F_{1,42} = 0.033$; $p = 0.858$), drug-treatment (interaction preference × drug-treatment: $F_{2,42} = 0.228$; $p = 0.797$), or an interaction between them (interaction preference × age × drug-treatment: $F_{2,42} = 0.228$; $p = 0.797$). Also, the entries into proximal or distal arms was not affected by drug-treatment (proximal arms: $F_{2,48} = 0.234$; $p = 0.792$; distal arms: $F_{2,48} = 0.103$; $p = 0.902$). Solely, there was an overall effect of age ($F_{1,42} = 4.646$; $p = 0.037$), while there was no overall effect of drug-treatment (main effect drug-treatment: $F_{2,42} = 0.186$; $p = 0.831$; interaction age × drug-treatment: $F_{2,42} = 0.434$; $p = 0.651$). Juvenile rats entered proximal and distal arms more often (0.50 ± 0.10 n/min) than adult rats (0.19 ± 0.10 n/min).

3.2.4. Playback of background noise—time spent on arms

Similarly, a comparison between the time spent in the proximal arms with that spent in distal arms, indicated a weak preference for the proximal arms in response to playback of background noise (main effect preference: $F_{1,42} = 6.248$; $p = 0.016$). During playback of background noise, rats spent 16.75 ± 3.37 s/min in the proximal arms, while only 6.45 ± 2.32 s/min in the distal ones. Again, it is important to note, however, that the preference towards the stimulus source in response to playback

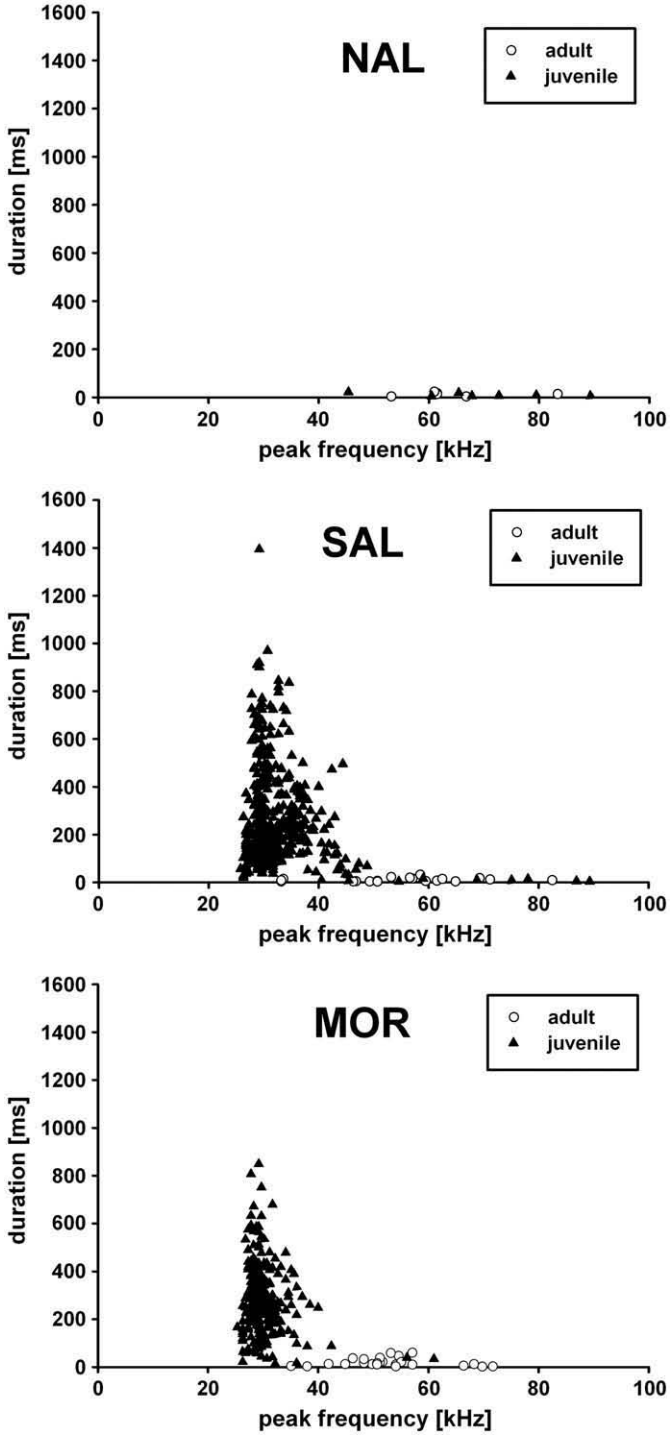

Fig. 4. Scatter plots depicting the distribution of ultrasonic vocalizations emitted during the entire experimental period by juvenile (black triangles) or adult (white circles) rats treated with NAL (naloxone; left graph), SAL (saline; middle graph), or MOR (morphine; right graph), plotted with respect to duration and peak frequency. Each dot reflects a single call.

of background noise was smaller than that one observed in response to playback of 50-kHz calls ($t_{47} = -3.124$; $p = 0.003$). Even more importantly, the preference for proximal arms during playback of background noise was not dependent on age (interaction preference × age: $F_{1,42} = 0.146$; $p = 0.704$), drug-treatment (interaction preference × drug-treatment: $F_{2,42} = 2.473$; $p = 0.096$), or an interaction between them (interaction preference × age × drug-treatment: $F_{2,42} = 0.307$; $p = 0.738$). Also, the time spent in proximal or distal arms was not affected by drug-treatment (proximal arms: $F_{2,48} = 2.210$; $p = 0.112$; distal arms: $F_{2,48} = 1.532$; $p = 0.228$). There was no an overall effect of age (main effect age: $F_{1,42} = 2.730$; $p = 0.106$) or drug-treatment (main effect drug-treatment: $F_{2,42} = 1.490$; $p = 0.237$; interaction age × drug-treatment: $F_{2,42} = 0.004$; $p = 0.996$).

3.2.5. Summary

Approach towards the stimulus source in response to 50-kHz calls, but not in response to background noise, was dependent on age and drug-treatment. Juvenile rats displayed a stronger approach behavior than adult rats. NAL treated rats showed the weakest response, while MOR treated rats showed the strongest. In comparison to SAL treated rats, approach was reduced in NAL treated rats, but enhanced in MOR treated rats.

3.3. Ultrasonic calling (Fig. 4 and Table 1)

3.3.1. Playback of 50-kHz ultrasonic vocalizations

Apart from behavioral changes, ultrasonic vocalizations were observed in response to playback of 50-kHz calls. Based on a scatter plot analysis, ultrasonic vocalizations were divided into calls with peak frequencies higher or lower than 45 kHz. In adult rats, both call types did not occur to a substantial amount, neither before, during nor after playback (all call numbers ≤ 1 calls/min). Also in juvenile rats, calls with peak frequencies higher than 45 kHz did not occur to a substantial amount, neither before, during nor after playback (all call numbers ≤ 1 calls/min). However, calls with peak frequencies lower than 45 kHz occurred to a substantial amount during (9.58 ± 5.97 calls/min) and after (3.07 ± 1.51 calls/min), but not before playback (call number ≤ 1 calls/min), in juvenile rats. When comparing the numbers of calls with peak frequencies lower than 45 kHz between drug-treatments in juvenile rats, a substantial amount of calls was seen in SAL (during playback: 25.38 ± 16.89 calls/min; after playback: 5.20 ± 2.39 calls/min) and MOR treated rats (during playback: 3.38 ± 3.38 calls/min; after playback: 4.00 ± 3.83 calls/min), but not in NAL treated rats (during playback: 00.00 ± 00.00 calls/min; after playback: 00.00 ± 00.00 calls/min). However, due to huge individual differences and hence a high level of variability, comparisons did not reach statistical significance (all p-values > 0.100; except main effect age: $F_{1,42} = 3.501$; $p = 0.068$).

3.3.2. Playback of background noise

Importantly, playback of background noise did not induce the emission of ultrasonic vocalizations. Both, in juvenile and adult rats, no substantial amount of calls with peak frequencies higher or lower than 45 kHz was observed, neither before, during nor after playback (all call numbers ≤ 1 calls/min).

3.3.3. Summary

Playback of 50-kHz calls, but not playback of background noise, led to the production of ultrasonic vocalizations with peak frequencies lower than 45 kHz in juvenile, but not adult rats. In juvenile rats, call production appeared to be drug-dependent. While a vocalization response was observed in SAL and MOR treated rats, no such response was observed in NAL treated rats.

4. Discussion

This is the first study demonstrating that the administration of pharmacological agents, namely opioid ligands, can affect social approach induced by playback of 50-kHz ultrasonic vocalizations. In line with our previous findings, playback of 50-kHz calls led to an increase in locomotor activity and approach towards the stimulus source in juvenile rats, and, to a lower extent, also in adult rats (Wöhr and Schwarting, 2007; Sadananda et al., 2008). Social approach is specific to 50-kHz calls, since it was not observed in response to background noise (present study; Wöhr and

Table 1
Ultrasonic calling.

	PRE-NOISE	NOISE	POST-NOISE	PRE-USV	USV	POST-USV
22-kHz calls						
NAL ($n=8$)	0.00 ± 0.00 (0)	0.00 ± 0.00 (0)	0.00 ± 0.00 (0)	0.00 ± 0.00 (0)	0.00 ± 0.00 (0)	0.00 ± 0.00 (0)
SAL ($n=8$)	0.00 ± 0.00 (0)	0.38 ± 0.38 (1)	0.23 ± 0.23 (1)	0.00 ± 0.00 (0)	25.38 ± 16.89 (5)	5.20 ± 2.39 (6)
MOR ($n=8$)	0.00 ± 0.00 (0)	2.63 ± 2.63 (1)	0.03 ± 0.03 (1)	0.00 ± 0.00 (0)	3.38 ± 3.38 (1)	4.00 ± 3.83 (2)
50-kHz calls						
NAL ($n=8$)	0.03 ± 0.03 (1)	0.00 ± 0.00 (0)	0.05 ± 0.03 (2)	0.00 ± 0.00 (0)	0.13 ± 0.13 (1)	0.03 ± 0.03 (1)
SAL ($n=8$)	0.03 ± 0.03 (1)	0.25 ± 0.16 (1)	0.03 ± 0.03 (1)	0.03 ± 0.03 (1)	0.63 ± 0.50 (2)	0.00 ± 0.00 (0)
MOR ($n=8$)	0.00 ± 0.00 (0)	0.00 ± 0.00 (0)	0.00 ± 0.00 (0)	0.00 ± 0.00 (0)	0.13 ± 0.13 (1)	0.05 ± 0.05 (1)

Numbers of the ontogenetic early form of 22-kHz ultrasonic vocalizations (upper part) and 50-kHz calls (lower part) in juvenile rats treated with NAL (naloxone), SAL (saline) or MOR (morphine). Values for test phases before (PRE-NOISE), during (NOISE) and after (POST-NOISE) playback of background noise are given on the left. Values for test phases before (PRE-USV), during (USV) and after (POST-USV) playback of 50-kHz calls are given on the right. The values before or after stimulus presentation reflect the averaged values measured during the respective five min periods. Values reflect means ± SEM per minute. Number of vocalizing rats per call type, drug-treatment, and test phase are given in parenthesis.

Schwarting, 2007; Sadananda et al., 2008) or 22-kHz calls, which induce locomotor inhibition (Brudzynski and Chiu, 1995; Burman et al., 2007; Sales, 1991; Wöhr and Schwarting, 2007) and activation of brain areas implicated in anxiety and fear (Sadananda et al., 2008), while social approach in response to 50-kHz calls was paralleled by an activation of brain areas implicated in reward processing such as the nucleus accumbens (Sadananda et al., 2008).

In juvenile rats, increases in locomotor activity and social approach in response to playback of 50-kHz calls were observed irrespective of drug-treatment. The strength of the response, however, was clearly dependent on drug-treatment. NAL treated rats showed the lowest level of social approach, while MOR treated rats showed the highest level of social approach. In fact, only MOR treated rats spent more time in the proximal arms during playback of 50-kHz calls than during playback of background noise. Furthermore, juvenile rats treated with SAL or MOR emitted ultrasonic vocalizations in response to playback of 50-kHz calls. Such ultrasonic calling was not seen in NAL treated rats.

In adult rats, increases in locomotor activity and social approach in response to playback of 50-kHz calls were also observed, but to a much lower degree than in juvenile rats. Despite the weakness of the response in adult rats, drug-treatment exerted effects in the same directions as in juvenile rats. Most importantly, no preference towards playback of 50-kHz calls was observed in NAL treated rats, while such preferences were seen in SAL and MOR treated rats. Again, MOR treated rats displayed the clearest preference towards the stimulus source, since only MOR treated rats spent more time in the proximal arms during playback of 50-kHz calls than during playback of background noise. Playback of 50-kHz calls did not induce substantial ultrasonic vocalization responses in adult rats.

The decreases or increases of social approach after treatment with NAL or MOR, respectively, parallels their effects on rough and tumble play. It was demonstrated that the μ-opioid-receptor-agonist MOR increases rough and tumble play, particularly after administration of 1 mg/kg (Niesink and Van Ree, 1989; Normansell and Panksepp, 1990; Panksepp et al., 1985; Siviy and Panksepp, 1985; Vanderschuren et al., 1995a, 1996). Similar effects were found with other μ-opioid-receptor-agonists such as methadone, fentanyl or beta-endorphin (Niesink and Van Ree, 1989; Vanderschuren et al., 1995b, 1997), while a decrease in rough and tumble play was observed after administration of μ-opioid-receptor-antagonists such as NAL (Beatty and Costello, 1982; Normansell and Panksepp, 1990; Panksepp et al., 1985; Siegel and Jensen, 1985, 1986; Siviy and Panksepp, 1985), naltrexone (Jalowiec et al., 1989; Niesink and Van Ree, 1989) or beta-funaltrexamine (Vanderschuren et al., 1995b). The similarity of the effects that μ-opioid-receptor-agonists and -antagonists exert on social approach induced by playback of 50-kHz calls and on rough and tumble play is remarkable. Thus, one can assume that the drug effects on rough and tumble play are at least partially due to their effects on responses to 50-kHz calls, which are abundantly detected during rough and tumble play (Burgdorf et al., 2008a; Brunelli et al., 2006; Knutson et al., 1998) or when rough and tumble play is mimicked by a human experimenter through tickling (Panksepp and Burgdorf, 2000, 2003; Schwarting et al., 2007). In fact, it was already shown that 50-kHz calling during rough and tumble play is an important feature of this behavior, since deafening or devocalizing affects it (Siviy and Panksepp, 1987). Furthermore, rats bred for high rates of 50-kHz calls display more rough and tumble play (Panksepp and Burgdorf, 2000). Interestingly, they also display an elevated level of met-enkephalin, an endogenous opioid, in various brain areas such as frontal cortex, septum, and hypothalamus (Burgdorf et al., 2008b).

Although the present findings fit nicely with what is known about the opioidergic regulation of rough and tumble play, they are in contrast to some studies on the effects of exogenous opiates on social behavior in other contexts. Panksepp et al. (1979) demonstrated that MOR reduces social cohesion, i.e. the time spent in proximity with other rats, as well as social locomotor activity, indicating that MOR decreases social motivation. This is in line with the hypothesis that activation of the endogenous opioid system triggers a feeling of social comfort, according to which injections of μ-opioid-receptor-agonists should decrease the need for gregariousness, while μ-opioid-receptor-antagonists should increase it (Panksepp et al., 1980). Despite the fact that this prediction was falsified in the context of rough and tumble play (Beatty and Costello, 1982; Jalowiec et al., 1989; Niesink and Van Ree, 1989; Normansell and Panksepp, 1990; Panksepp et al., 1985; Siegel and Jensen, 1985, 1986; Siviy and Panksepp, 1985; Vanderschuren et al., 1995a,b,c, 1996, 1997), there are some studies, which support it. Thus, Panksepp et al. (1980) anecdotally reported that NAL increases the preference for a social reward while MOR enhances the preference for a food reward. Furthermore, MOR reduced social investigation in a social-hole-task (Deak et al., 2009), and another μ-opioid-receptor-agonist, methadone, was shown to lower levels of social locomotor activity, time spent in social contact, and grooming each other (Plonsky and Freeman, 1982). Also, MOR withdrawal increased social interest, suggesting that "prolonged opiate abstinence is a condition which may enhance social rewards, perhaps in an attempt to re-establish endogenous opioid homeostasis" (Nocjar and Panksepp, 2007, p. 195). However, in some of these studies inconsistent results were obtained. For instance, although MOR decreased social cohesion, no clear induction of social cohesion was observed after treatment with NAL (Panksepp et al., 1979). Similarly, MOR decreased the time spent in social investigation in the social-hole-task, but also naltrexone (Deak et al., 2009). Finally, there are also studies where drug effects were observed in the same directions as in studies on rough and tumble play. Specifically, File (1980) found that Long–Evans hooded rats that received 2 mg/kg NAL showed decreased social locomotor activity and reduced social interaction, i.e. they spent less time interacting with other rats. DeRossett and Holtzman (1982) reported decreased social locomotor activity in Sprague–Dawley rats after administration of various doses of NAL. More recently, Dokla (1992) confirmed these findings in a detailed study, where it was found

that 1 or 4 mg/kg NAL reduced social locomotor activity in Long–Evans hooded rats. All these latter studies are in line with the present results.

Several possible mechanisms have been suggested why MOR is fostering social interactions, while NAL is hindering it. Among others, it was hypothesized that MOR enhances rough and tumble play by increasing its rewarding value (Vanderschuren et al., 1995a). This hypothesis is based on two observations. Firstly, rough and tumble play is clearly rewarding for juvenile rats. Thus, juvenile rats learn tasks such as spatial discrimination to obtain the opportunity to play (Humphreys and Einon, 1981; Normansell and Panksepp, 1990). Secondly, the opioid system is implicated in reward processes (Van Ree et al., 2000; Wise, 1989). In fact, there is some experimental evidence for this hypothesis. Although both MOR and NAL treated animals learned similarly fast to establish a preference for a context associated with the opportunity to play, MOR treated rats were more resistant to extinction than NAL treated rats (Normansell and Panksepp, 1990). Moreover, NAL reduced rough and tumble play in rats by reducing the frequency of play bouts without affecting their duration, indicating that NAL is reducing the rewarding value of rough and tumble play (Beatty and Costello, 1982).

Another hypothesis is that an activation of the opioid system increases rough and tumble play by promoting a state of social comfort and thus assertiveness (Vanderschuren et al., 1997). In fact, it was repeatedly shown that central or peripheral injections of MOR or several other μ-opioid-receptor-agonists can diminish isolation-induced distress vocalizations in various species such as monkeys (Kalin et al., 1988), chicks (Panksepp et al. 1978a,b; Sufka et al., 1994; Vilberg et al., 1984), guinea pigs (Herman and Panksepp, 1978), mice (Moles et al., 2004), or rats (Carden and Hofer, 1990a,b; Kehoe and Blass, 1986), while the μ-opioid-receptor-antagonists, NAL or naltrexone, block these opioid effects and increase the number of isolation-induced vocalizations when given alone (Carden and Hofer, 1990a,b; Herman and Panksepp, 1978; Kalin et al., 1988; Kehoe and Blass, 1986; Sufka et al., 1994). This hypothesis is further supported by the observation that MOR treatment can make submissive rats more dominant, while NAL treatment can make dominant rats more submissive (Panksepp et al., 1985). Finally, it was shown that an initial, novelty-induced suppression of rough and tumble play can be abolished by MOR at doses, which do not affect rough and tumble play in familiar environments (Vanderschuren et al., 1995a).

The present findings are compatible with both hypotheses. Thus, MOR might have increased the incentive value of 50-kHz calls, while NAL might have reduced it. An incentive value of 50-kHz calls is not only indicated by the strong approach displayed in repose to such calls (present study; Wöhr and Schwarting, 2007), but also by a recent study by Burgdorf et al. (2008a), where it was found that rats self-administer playback of 50-kHz calls, but not tape hiss or 22-kHz calls. MOR might also have increased assertiveness and thus social approach, while NAL might have had opposite effects. However, this latter possibility seems to be less likely for two reasons. Firstly, the dose where Vanderschuren et al. (1995a) observed the induction of assertiveness was 10 times lower than the present one, a dose which mainly affected the rewarding values of rough and tumble play in the study by Vanderschuren et al. (1995a). Secondly, the rats tested in the present study were habituated to the test environment for 15 min before stimulus presentation, whereas the effects on assertiveness were detected in the very first minute of testing (Vanderschuren et al., 1995a).

In total, the present data on the effects of opioid ligands on the behavioral responses to playback of 50-kHz calls indicate that an important feature of social interaction in rats, namely ultrasonic communication, is at least partially regulated by endogenous opioids, probably by mediating changes in the incentive value of 50-kHz calls.

Apart from these behavioral changes, playback of 50-kHz calls, but not of background noise, did also induce ultrasonic calling. In line with our previous studies (Wöhr and Schwarting, 2007; Sadananda et al., 2008), very low rates of ultrasonic vocalizations were detected in adult rats. More importantly, however, and for the first time, a substantial amount of ultrasonic vocalizations in response to playback of 50-kHz calls was detected in juvenile rats. This finding does not only underscore the possible communicative function of 50-kHz calls, but has also important interpretative implications. It is remarkable that the decrease in call emission from adolescence to adulthood paralleled the decline in social approach. Recently, Salchner et al. (2004) found that aged rats spent considerably less time in active social interaction than young rats. Moles et al. (2007) observed similar changes as a function of age during social interaction of mice. Aged mice did not only show a decrease in the time investigating conspecifics, but also in the number of ultrasonic vocalizations. Therefore, one might interpret the present decline in both, social approach and ultrasonic calling, as the result of a decrease in social interest as a function of aging. However, it has to be noted that the lower response in adult rats could also depend on the stimulus used. Here, we used 50-kHz calls recorded from an adult male Wistar rat during exploration of a cage containing scents from a cage mate (Wöhr et al., 2008). A different result might be obtained with 50-kHz calls recorded in other contexts. In fact, playback of 50-kHz calls recorded during tickling or exploration of an empty clean cage had no or only minor effects on the behavior of the recipient (Burman et al., 2007; Endres et al., 2007), and there is evidence showing that call characteristics of 50-kHz ultrasonic vocalizations vary depending on the social context (Burgdorf et al., 2008a).

The main proportion of ultrasonic vocalizations emitted by juvenile rats in response to playback of 50-kHz calls falls into the class of an ontogenetic early form of 22-kHz calls. Blanchard et al. (1990) anecdotally reported that 22-kHz calls of juvenile rats are characterized by an untypical high peak frequency of 32 kHz and we were able to demonstrate that peak frequency declines, while call duration increases, as a function of age (Wöhr and Schwarting, in preparation). When accepting that juvenile rats emitted an ontogenetic early form of 22-kHz calls, the question raises, why would juvenile rats produce 22-kHz calls while displaying approach? It is widely accepted that 22-kHz calls reflect a negative affective state, since they occur in aversive situations such as social defeat (Burgdorf et al., 2008a; Frank et al., 2006; Sales, 1972a; Thomas et al., 1983), predator exposure (Blanchard et al., 1990, 1991, 1992; Shepherd et al., 1992), and after delivery of electric shock (Borta et al., 2006; Choi and Brown, 2003; Molewijk et al., 1995; Van der Poel et al., 1989; Wöhr and Schwarting, 2008a,b; Wöhr et al., 2005). Therefore, it appears possible that juvenile rats emitted 22-kHz calls because of fear for the presence of an unknown adult male rat (Sales, 1972a). Furthermore, one would expect that the production of 22-kHz calls is positively associated with avoidance behavior such as freezing (Choi and Brown, 2003; Wöhr and Schwarting, 2008a,b; Wöhr et al., 2005). However, as in our recent studies (Wöhr and Schwarting, 2007; Sadananda et al., 2008), social approach rather than avoidance was observed in response to playback of 50-kHz calls. Moreover, in contrast to what one might have expected based on the current knowledge on 22-kHz calls, the emission of 22-kHz calls was observed in rats, which tried most vigorously, although without success, to reach the stimulus source, namely juvenile rats treated with SAL or MOR. Juvenile rats treated with NAL or adult rats, both displaying much lower levels of social approach, did not emit 22-kHz calls. Therefore, the present results indicate that the occurrence of 22-kHz calls might be due to the fact that an incentive stimulus, i.e. 50-kHz calls, was not followed by the appropriate rewarding stimulus, i.e. a social partner, and thus might reflect a state of frustration. It is in line with this hypothesis that unexpected termination of rewarding stimuli, such as electrical brain stimulation or food, induced the emission of 22-kHz calls (Burgdorf et al., 2000). Burgdorf et al. (2000) suggested that a similar dynamic may underlay the production of 22-kHz calls during withdrawal from opiates or psychostimulants (Barros and Miczek, 1996; Covington and Miczek, 2003; Mutschler and Miczek, 1998; Vivian and Miczek, 1991).

In this context it is important to note that the present drug effects were specifically seen in response to playback of 50-kHz calls. There were no acute drug effects on call production before playback of 50-kHz calls, i.e. no induction of ultrasonic vocalizations by NAL or MOR.

This is in line with what is known from the literature. Although, Burgdorf et al. (2001b) reported that NAL can induce 22-kHz calling, their effects were seen after administration of the rather high dosage of 3 mg/kg, and hence probably due to aversive drug effects. In fact, a dose of 1 mg/kg did not affect ultrasonic calling during tickling (Panksepp and Burgdorf, 2000) or inconsistent results were obtained (Burgdorf and Panksepp, 2001). However, on the basis of the literature on drugs of abuse, such as the psychostimulant amphetamine, which can induce high rates of 50-kHz calls (Burgdorf et al., 2001a; Natusch et al., 2008; Thompson et al., 2006; Wintink and Brudzynski, 2001), one could expect that also MOR would induce 50-kHz calling. In line with the present results, however, there is no clear evidence that MOR can induce ultrasonic calling. Knutson et al. (1999) as well as Burgdorf et al. (2001b) reported 50-kHz calls only in anticipation of MOR, but not as an acute effect of the drug. Moreover, Panksepp and Burgdorf (2000) did not observe an effect of MOR on the emission of 50-kHz calls during tickling. So far, only Tyr-D-Ala-Gly-N-methyl-Phe-Gly-ol (DAMGO), a μ-opioid-receptor-agonist, was shown to increase the rate of 50-kHz calls when injected centrally into the ventral tegmental area (Burgdorf et al., 2007). Finally, also for psychostimulants the picture is not as clear as one would expect based on the strong effects of amphetamine, since other psychostimulants, such as 3,4-methylenedioxy-*N*-methylamphetamine (MDMA, "ecstasy"), did not induce 50-kHz calling in a recent study by Natusch et al. (2008), indicating that the production of 50-kHz calls after administration of amphetamine might be a rather specific phenomenon.

In total, the present data on the effects of opioid ligands on the vocal responses to playback of 50-kHz calls are in line with the present behavioral data. In general, the finding that the administration of opioid ligands can affect social approach and ultrasonic vocalizations induced by playback of 50-kHz calls, indicates that an important feature of social interaction in rats, namely ultrasonic communication, is at least partially regulated by endogenous opioids, probably by mediating changes in the incentive value of 50-kHz calls. This shows that the measurement of social approach in response to playback of 50-kHz calls is sensitive for bidirectional changes in the level of social interest induced by pharmacological agents, offering the opportunity to screen various pharmacological agents implicated in the regulation of social interest, which might help to identify treatments for neuropsychiatric disorders with social deficits such as autism.

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