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ORIGINAL INVESTIGATION

Examining face and construct validity of a noninvasive model of panic disorder in Lister-hooded rats

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Abstract
Rationale Increasing evidence suggests that defensive escape behavior in Lister-hooded (LH) rats induced by ultrasound application may be an animal model of panic disorder.
Objective The objectives of this study were to further explore the face and construct validity of ultrasound-induced escape behavior by characterizing the autonomic and neuroendocrine response to ultrasound, and to examine the underlying neuronal structures by comparing the effects of the anxiolytic with panicolytic properties, diazepam, with a preclinical anxiolytic without panicolytic-like activity, the NOP agonist Ro 64-6198.
Materials and methods LH rats were implanted with telemetry transmitters to monitor heart rate and core body temperature before, during, and after ultrasound application. Blood samples were taken after ultrasound application for corticosterone analysis. Ultrasound-induced c-Fos expression was measured in different periaqueductal gray (PAG) and amygdala subregions after treatment with diazepam or Ro 64-6198.
Results Ultrasound application increased heart rate and body temperature, but did not alter plasma corticosterone levels. Ultrasound application increased c-Fos expression in the dorsal and dorsolateral PAG (dPAG, dlPAG) and amygdaloid subregions. Diazepam, but not Ro 64-6198, reduced c-Fos expression in the dPAG/dlPAG, while Ro 64-6198, but not diazepam, reduced c-Fos expression in the central amygdala.
Conclusions Similar to human panic attacks, ultrasound application to LH rats activated the autonomic, but not the neuroendocrine, stress system. Also, like in humans, the current data confirm and extend that the dPAG/dlPAG plays a key role in ultrasound-induced escape behavior. These observations suggest that ultrasound-induced escape behaviors in LH rats have face and construct validity for panic disorders.

Keywords Ultrasound · Panic · Periaqueductal gray · Amygdala · Heart rate · Core body temperature · Corticosterone · c-Fos · PAG stimulation

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Introduction

Panic disorder is characterized by spontaneously occurring panic attacks, which typically include a sudden experience of intense distress and severe anxiety, often accompanied by a block while walking and/or a desire to flee. These emotional feelings are accompanied by autonomic responses such as

dyspnea, hypertension, palpitation, sweating, or dizziness (DSM IV 2003; Goetz et al. 1994; Schenberg et al. 2001).

With respect to the underlying mechanism, brain imaging studies of panic disorder patients experiencing an acute panic attack display increased activity in the periaqueductal gray (PAG), the amygdala, and the superior colliculi (Boshuisen et al. 2002; Javanmard et al. 1999; reviewed in Graeff and Del-Ben 2008; Del-Ben and Graeff 2009). Additionally, electrical stimulation of the dorsolateral PAG (dlPAG) in awake humans who do not suffer from panic disorder induces emotional and autonomic responses which appear to resemble spontaneously occurring panic attacks (Iacono and Nashold 1982).

In rats, electrical or chemical stimulation of the dorsal regions of the PAG (dPAG and dlPAG) induce freezing and, with increasing intensity, escape responses (summarized in Schenberg et al. 2001). These behaviors are similar to those naturally occurring in rodents in a potentially threatening situation (e.g., when faced with a natural predator species; Blanchard et al. 1986) and are accompanied by autonomic responses including tachycardia and hyperventilation, which are thought to resemble symptoms of human dlPAG stimulation and spontaneously occurring panic attacks (summarized in Schenberg et al. 2001). Since the dlPAG stimulation model is sensitive to panicolytic drugs, it is widely used and considered as perhaps the best characterized preclinical animal model for panic disorder (e.g., Jung et al. 2001; Graeff et al. 1986; Graeff 1994, 2004; Jenck et al. 1995; Brandão et al. 1982, 1994).

Beckett et al. (1996) examined an alternative way to stimulate the dorsal regions of the PAG in Lister-hooded (LH) rats in a noninvasive manner, by applying 22-kHz ultrasound. Rats use 22-kHz ultrasonic vocalizations as alarm calls, and it has been previously shown that such alarm calls can induce defensive behaviors in conspecifics (Blanchard et al. 1991; Brudzynski and Chiu 1995). Accordingly, Beckett et al. (1996, 1997) observed that applying artificial 22-kHz ultrasounds at intensities of at least 65 dB induced escape and freezing behaviors in LH, but not in Wistar rats, which showed only increased freezing behavior (Beckett et al. 1996; Neophytou et al. 2000). Interestingly, ultrasound-induced defensive behaviors correlated with increased c-Fos expression in the dorsal regions of the PAG, the amygdala, and in thalamic and hypothalamic nuclei, i.e. brain areas associated with aversive behaviors (Neophytou et al. 2000).

In a recent study, we demonstrated that ultrasound-induced defensive behaviors in LH rats can be modulated by different anxiolytics (Nicolas et al. 2007). For example, diazepam and chlordiazepoxide, two benzodiazepines with known clinical efficiency in panic disorder (e.g., Noyes et al. 1996; Spiegel and Bruce 1997), and gabapentin, an anticonvulsant and anxiolytic which is thought to have panicolytic properties (Layton et al. 2001; Pande et al. 2000; reviewed in Van Ameringen et al. 2004), selectively reduced escape response during ultrasound application. On the other hand, anxiolytics without panicolytic action, like buspirone (reviewed in Chessick et al. 2006) had no effect on ultrasound-induced behaviors. In contrast to all other drugs tested in this study, the nociceptin orphanin FQ peptide (NOP) receptor agonist Ro 64-6198, which produces broad anxiolytic effects in preclinical models (Jenck et al. 2000; Varty et al. 2005), selectively reduced freezing but not escape behaviors. This makes it an interesting tool compound with which to probe the different neuronal mechanisms that are responsible for escape versus freezing responses (Nicolas et al. 2007). Taken together, ultrasound-induced escape seems to have high predictive validity for panic disorder, although the sensitivity to chronic treatment with antidepressants remains to be established.

The present study investigates aspects of face and construct validity. Face validity requires similarities in behavioral or physiological reactions in humans and animals. Since increased heart rate is an autonomic symptom of panic attacks (DSM IV 2003), the heart rate response of LH rats to ultrasound was measured with telemetry. In addition, core body temperature was measured as despite unclear effects of a panic attack in humans on this parameter, preclinical studies have shown that hyperthermia is an important physiological marker of stress-induced autonomic activation (Vidal et al. 1984; Olivier et al. 2003). Another parameter of interest with respect to face validity is blood corticosterone level because even though panic attacks are experienced as highly stressful events, patients do not show an increase in cortisol levels after an acute panic attack, and the same is true for corticosterone levels in rodents after stimulation of the dorsal regions of the PAG (reviewed in Graeff et al. 2005; Graeff 2007). Therefore, we quantified plasma corticosterone levels of LH rats after ultrasound application. Construct validity requires a similarity in underlying mechanisms and/or brain structures in the clinical situation and in the animal model. We confirmed that ultrasound-induced defensive behaviors correlate with increased c-Fos expression in the PAG and the amygdala and examined whether or not this expression pattern is differentially modulated by diazepam and Ro 64-6198, i.e., two compounds that produce opposite behavioral profiles in this model (Nicolas et al. 2007), allowing us to compare regional brain activity and specific behavioral patterns presented in this model.

Materials and methods

Animals

Male LH rats (Harlan, Netherlands, 220–250 g; $n=64$) were housed in groups ($n=4$ per cage) and acclimated for 5–

7 days before the start of test or habituation. They were housed with sawdust bedding under standard maintenance conditions (12:12-h light–dark cycle, 21–23°C, 55–65% relative humidity). Food and water were given ad libitum in the home cage. All testing was performed during the light phase of the light–dark cycle. Animals were exposed to ultrasound only once and were killed by CO_2 exposure after testing, with the exception of the animals used for the c-Fos study which were deeply anesthetized and transcardially perfused with an extemporaneous 4% paraformaldehyde in 0.1 mol/L phosphate buffer (PB), pH 7.4, solution. The present procedure was approved by the Basel Stadt Kantonal Veterinary Authority based on adherence to Swiss federal regulations and guidelines on animal experimentation provided by the Swiss Academy of Sciences and Swiss Academy of Medical Sciences (1995).

Drugs

Diazepam (3 mg/kg body weight) and Ro 64-6198 (10 mg/kg; all drugs synthesized at F. Hoffmann, La Roche Ltd.) were administered in 0.3% Tween-80 in physiological saline (0.9% NaCl) in a volume of 5 ml/kg body weight. Diazepam and Ro 64-6198 were injected i.p. with a pretreatment time of 30 min.

The given drugs and doses were chosen on the basis of former experiments which revealed that these doses produced significant effect on ultrasound induced escape (diazepam) or freezing (Ro 64-6198) behavior in the absence of a significant effect on baseline locomotion (Nicolas et al. 2007). Other studies (e.g., Gomita et al. 1991) have shown that diazepam significantly increased the threshold needed for electrical dPAG stimulation-induced escape response at doses from 2–10 mg/kg. Similar to clinical situations (Kasper and Resinger 2001), doses of diazepam necessary to reduce panic-related behavior in these setting are higher than those routinely used to reduce anxiety-like behavior in rodent exploratory models (typically 1–2 mg/kg; for an example, see Pellow et al. 1985).

Apparatus

Artificially generated ultrasound (22 kHz, 93 dB) was applied for a duration of 1 min in a computer-controlled fear conditioning system (TSE, Bad Homburg, Germany), which was modified by the supplier to allow the generation of ultrasound. Therefore, calibrated high-frequency loudspeakers were mounted on the ceiling of each sound-attenuating outer box. The test arenas were made of transparent Perspex walls ($L \times H \times W$, 45×45×45 cm) with gray plastic floors. They were placed in the outer boxes (not actively ventilated) and were constantly and evenly illuminated by a white ceiling light (25 lux). For the telemetry experiments, we placed the telemetry antennas (DSI, RPC1 Physio Tel receivers) under the test apparatus, allowing us to monitor heart rate and core body temperature of the test animals. Additionally, the telemetry system provides a crude measurement of the animals' general activity. After each test, the boxes were carefully cleaned with 70% ethanol.

Procedures

Telemetry

The animals used for the telemetry study were intraperitoneally implanted with telemetry transmitters (DSI, CTA F-40), allowing the monitoring of heart rate, core body temperature, and locomotor activity. Data were sampled by a computer every 10 s (DSI DATAQUEST A.R.T. software).

Since pilot studies showed that the animals used for telemetry studies needed more than 1 h to reach a stable baseline in heart rate and core body temperature when they were placed into the test boxes for the first time, the animals used for this experiment were habituated daily (up to approx. 1 week) to the test cage until they reached a stable baseline heart rate and core body temperature within approx. 30 min. On the test day, untreated animals (n=6) were placed into the test boxes 45 min before ultrasound application to allow heart rate and core body temperature to reach stable baseline before ultrasound application. After the ultrasound application, they remained in the test box for another hour to monitor the heart rate and core body temperature. There was one animal in which the transmitter did not produce reliable values for heart rate, and in one animal, activity was not monitored, so we used only five animals for statistical analysis of heart rate and locomotor activity, but six for the analysis of core body temperature.

Corticosterone measurements

For corticosterone measurements, we first used non-habituated rats (n=24) that were placed into the test boxes 3 min before ultrasound application, in accordance with our normal ultrasound testing protocol (Nicolas et al. 2007). Rats that were placed into the test boxes without ultrasound application served as controls in this experiment. In addition, to compare test conditions with home conditions, another group of rats was left undisturbed in their home cages (n=8 for all groups). In a second experiment, in an attempt to make this experiment more comparable to the telemetry study, we tested two additional groups of rats (n=8 for each group) in the same manner as above, but with the difference that they were exposed to the test boxes for 1 h daily for 3 days before testing and that on the test

day, they were placed into the boxes 45 min, instead of 3 min, before ultrasound application.

The liquid chromatography–tandem mass spectrometry system (LC/MS-MS) consisted of a CTC PAL autosampler, a Shimadzu LC10 high-pressure gradient system, an eluent diverting valve, and a Waters Quattro Ultima triple quadrupole mass spectrometer. The HPLC column was a 2×150 mm Inertsil ODS3 column with 5 μm of particle size operated at room temperature.

Sample processing

Rats were decapitated 25 min after completion of the experiment. Blood was collected in EDTA-coated 1.5-ml Eppendorf cups, centrifuged for 5 min at 4,000 rpm and 4°C; plasma supernatant was transferred to another Eppendorf cup. Samples were then kept at −80°C until further processing.

In a 2-ml Eppendorf vial, 100 μl of plasma was spiked with internal standard solution (D_8-corticosterone; CDN, Quebec, Canada) and extracted twice with 1.2 ml of butyl chloride (Fluka, Switzerland) containing 4% of dichloromethane (Fluka). The phases were separated by centrifugation, freezing of the aqueous layer, and transfer of the organic phases to a second Eppendorf vial where they were evaporated to dryness. The residue was dissolved in 100 μl of 20% methanol (Fluka)/80% water, and 10 μl was injected to the LC/MS-MS system.

Analytical operating conditions

The HPLC gradient conditions were water–methanol 68% to 85% methanolic in 6 min, 200 μl/min, runtime 10 min. The LC effluent was only switched to the mass spectrometer during peak elution. The mass spectrometer was operated in positive ion electrospray mode with nitrogen as desolvating and spraying gas. The accurate decimals of the masses were determined by a specific optimization experiment. The ions of the M+Na+MeOH adducts at *m/z* 401 (corticosterone) and *m/z* 409 (D_8-corticosterone) were recorded in single ion monitoring mode and 200 ms of dwell time. With this unusual operational mode, we obtained a better S/N ratio than with MS/MS due to the inefficient fragmentation behavior of the steroids and the distribution of the signal over MH^+, MNa^+, and methanol adducts. The quantification limit is around 5 ng/ml.

Calibration and data analysis

The calibration samples were prepared by spiking blank rat plasma containing low levels of corticosterone with up to 1,000 ng/ml of corticosterone. The resulting calibration plot has an intercept which reflects the concentration of corticosterone in the blank plasma. To calculate *absolute* concentrations, the peak area ratio was divided by the slope of the calibration line.

c-Fos study

The animals were injected with either vehicle ($n=4$), diazepam ($n=4$), or Ro 64-6198 ($n=5$) 30 min before they were put into the test cages. Ultrasound was then applied after 3 min of habituation, according to the normal test protocol. Animals which received a vehicle injection and were placed into the boxes without ultrasound application served as controls ($n=5$).

Sixty minutes after termination of the behavioral experiment, animals were deeply anesthetized with sodium pentobarbital and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde in 0.1 mol/L PB, pH 7.4. Brains were then removed and post-fixed at 4°C overnight in 4% paraformaldehyde in PB. Fifty-micrometer sections were coronally cut with a Leica vibratome and collected in immunobuffer. Six to eight sections per animal and for each studied area were used for cell counts. The sections were processed for c-Fos immunoreactivity as previously described (Zhang et al. 2002). Antibody incubations were performed in PB 0.1 M with 0.3% triton X-100 and 0.3% bovine serum albumin. Sections were blocked for 30 min in a solution with 10% methanol and 3% hydrogen peroxide. A rabbit anti-c-Fos antibody (Santa Cruz Biotechnology, Inc.) 1:1,000 was used to detect the nucleus of activated neurons. The secondary antibody used was a biotinylated mouse anti-Rabbit IgG (Santa Cruz Biotechnology, Inc.) 1:1,000 followed by the peroxidase-based ABC system (Vector) using diaminobenzidine as the chromogen. All sections were mounted onto gelatin-coated microscope slides. Morphometrical analyses were performed as described (Lopez-Lopez et al. 2004). In brief, c-Fos-positive cells were quantified using an unbiased stereological method with the optical dissector (Reed and Howard 1998) counted by an observer blinded to the experimental treatment. Only cells containing a nuclear brown reaction product were considered to be c-Fos-positive. The anatomical localization of c-Fos-positive cells was aided by using the illustrations in a stereotaxic atlas (Paxinos and Watson 1998). To determine cell density, the counting area was drawn using a digital camera (PixelFly, Berlin, Germany). The area was then estimated using the point-counting method of Weibel (Weibel et al. 1966; Weibel 1979).

Statistics

All statistical analyses were carried out with the statistic software SigmaStat (Statcon, Witzenhausen, Germany). All

data are expressed as mean + SEM. Significance was set at $p \leq 0.05$.

The mean heart rate, mean core body temperature, and mean locomotor activity were calculated for every 5-min interval and analyzed by one-way repeated measure analysis of variance (ANOVA) followed by Tukey's multiple comparison test which was used to compare each interval to the interval before ultrasound application (data point 0; Fig. 1).

Plasma corticosterone levels were analyzed by nonparametric Mann–Whitney rank sum tests, comparing the animals which received ultrasound application to the corresponding control group that did not receive ultrasound application.

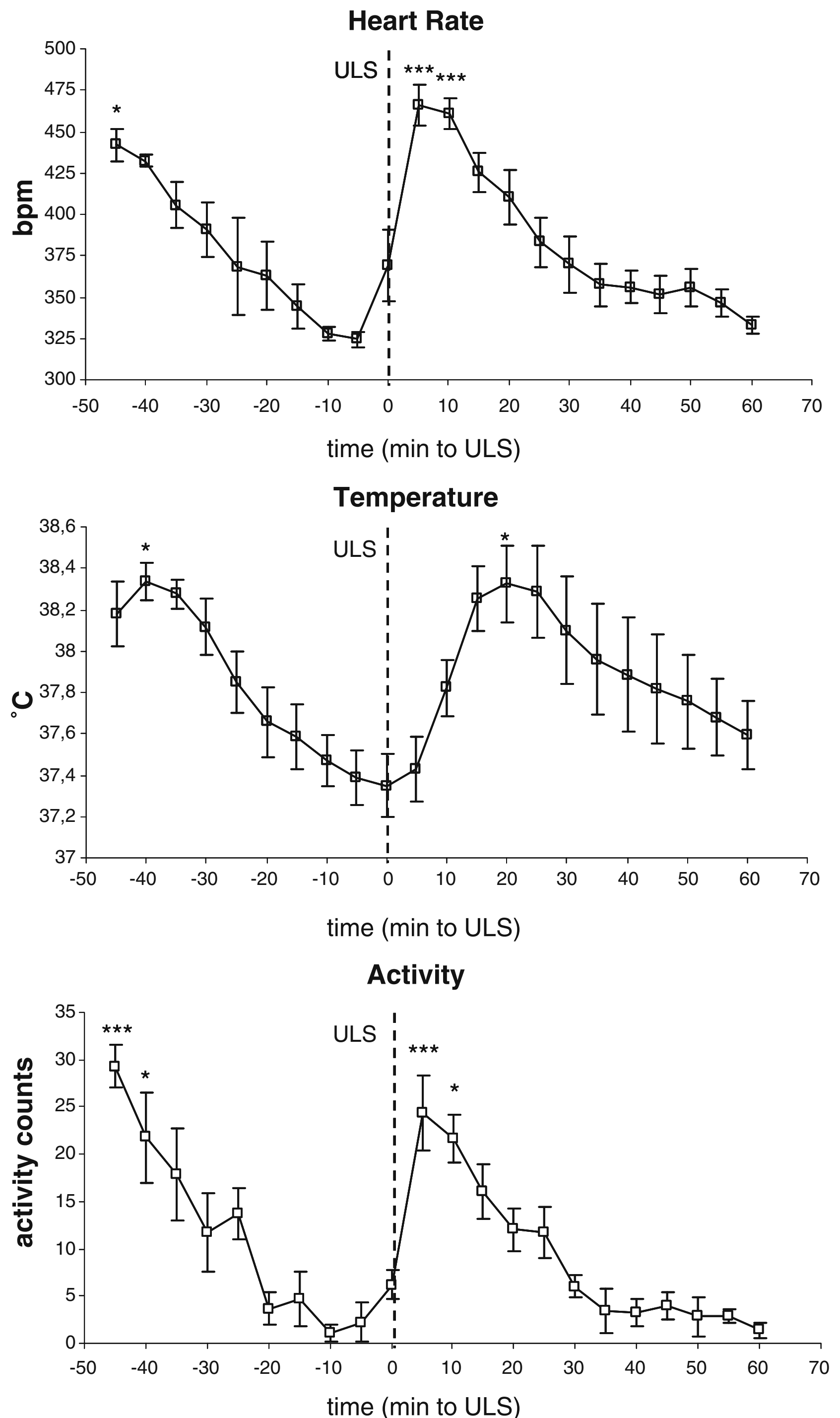

Fig. 1 Telemetry data of a ~2-h test session (mean ± SEM). Ultrasound (*ULS*) was applied for 1 min after a habituation time of 45 min (time interval 0) and induced an increase in heart rate (**a**) (n=5), body temperature (**b**) (n=6), and locomotor activity (**c**) (n=5). *Asterisks* indicate significant differences to time interval 0 (one-way ANOVA, followed Tukey's post hoc testing comparing each interval with the one before ULS application (data point 0). *$p<0.05$; ***$p<0.001$)

Numbers of c-Fos immunoreactive cells within the different subregions of the PAG and the amygdala were analyzed by one-way ANOVA, followed by Tukey's multiple comparison procedure.

Results

Telemetry

After several habituation trials, all animals habituated to the boxes and showed normalization of heart rate and core body temperature within 30 min. On the testing day, all animals reached normal values for heart rate (below 350 bpm) and temperature (below 37.5°C; Fig. 1), comparable to the last habituation trial (data not shown).

Ultrasound produced a marked increase in all three parameters. For heart rate, one-way repeated measure ANOVA revealed significant differences between the different time intervals ($F(21,84)=10.80$, $p<0.001$). Tukey's multiple comparison test revealed that compared to the last interval before ultrasound application (data point 0), heart rate was significantly higher 45 min before and 5 and 10 min after ultrasound application ($p=0.022$, $p<0.001$, $p<0.001$, respectively; Fig. 1a).

For core body temperature, one-way repeated measure ANOVA revealed significant differences between the different time intervals ($F(21,105)=6.75$, $p<0.001$). Tukey's multiple comparison test revealed that compared to the last interval before ultrasound application (data point 0), core body temperature was significantly higher 40 min before and 20 min after ultrasound application ($p=0.034$ and $p<0.039$, respectively; Fig. 1b).

For activity, one-way repeated measure ANOVA revealed significant differences between the different time intervals ($F(21,84)=10.11$, $p<0.001$). Tukey's multiple comparison test revealed that compared to the last interval before ultrasound application (data point 0), activity was significantly higher 45 and 40 min before and 5 and 10 min after ultrasound application ($p<0.001$, $p=0.013$, $p=0.001$, $p=0.013$, respectively; Fig. 1c).

It should be noted that the present study did not examine the detailed behavior during (i.e., wild running followed by freezing) and just after (i.e., freezing) the ultrasound application; for a description of these behaviors, see Nicolas et al. (2007).

Plasma corticosterone levels

The application of ultrasound resulted in a remarkable absence of effects on plasma corticosterone levels both in the non-habituated animals and in the habituated animals, Fig. 2 (both $p>0.5$). Opposite to our expectations, the habituated control group showed higher corticosterone levels than the non-habituated control animals, although this did not reach significance ($p=0.083$). The corticosterone levels in the homecage animals were significantly lower as compared to all other groups (21.04±7.59 ng/ml, $p<0.05$, Fig. 2).

c-Fos

After application of ultrasound, the number of c-Fos immunoreactive cells was increased in several subregions of the PAG and the amygdala. In the different subregions, this increase was differentially modulated by diazepam or Ro 64-6198 pretreatments.

Dorsal PAG One-way ANOVA revealed significant differences in the number of c-Fos-positive cells after the different treatments with or without ultrasound ($F(3,14)=21.26$, $p<0.001$). Tukey's multiple comparison test revealed a significant increase in c-Fos-positive cells in the dorsal PAG after ultrasound application in animals treated with vehicle ($p<0.01$) or Ro 64-6198 ($p<0.05$). In contrast, a decrease was observed in diazepam-treated animals ($p<0.05$). In addition, the number of c-Fos-positive cells after ultrasound application was significantly lower in diazepam-treated animals when compared to vehicle-treated animals ($p<0.001$; Fig. 3a).

Dorsolateral PAG One-way ANOVA revealed significant differences in the number of c-Fos-positive cells after the different treatments with or without ultrasound ($F(3,14)=21.43$, $p<0.001$). Tukey's multiple comparison test revealed a significant increase in c-Fos-positive cells after ultrasound application in animals treated with vehicle ($p<0.01$) or Ro 64-6198 ($p<0.05$), whereas a decrease was found in diazepam-treated animals ($p<0.05$). In addition, the number

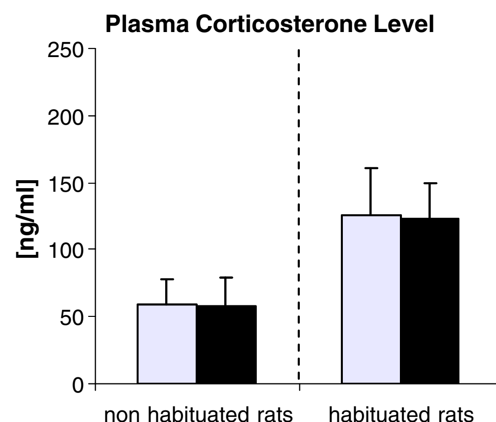

Fig. 2 Plasma corticosterone levels (mean + SEM) of non-habituated and habituated animals put into the test arena with (*black bars*) or without (*white bars*) ultrasound ($n=8$ each). Non-parametric Mann–Whitney rank sum tests revealed no significant differences between animals which received ultrasound and their corresponding controls

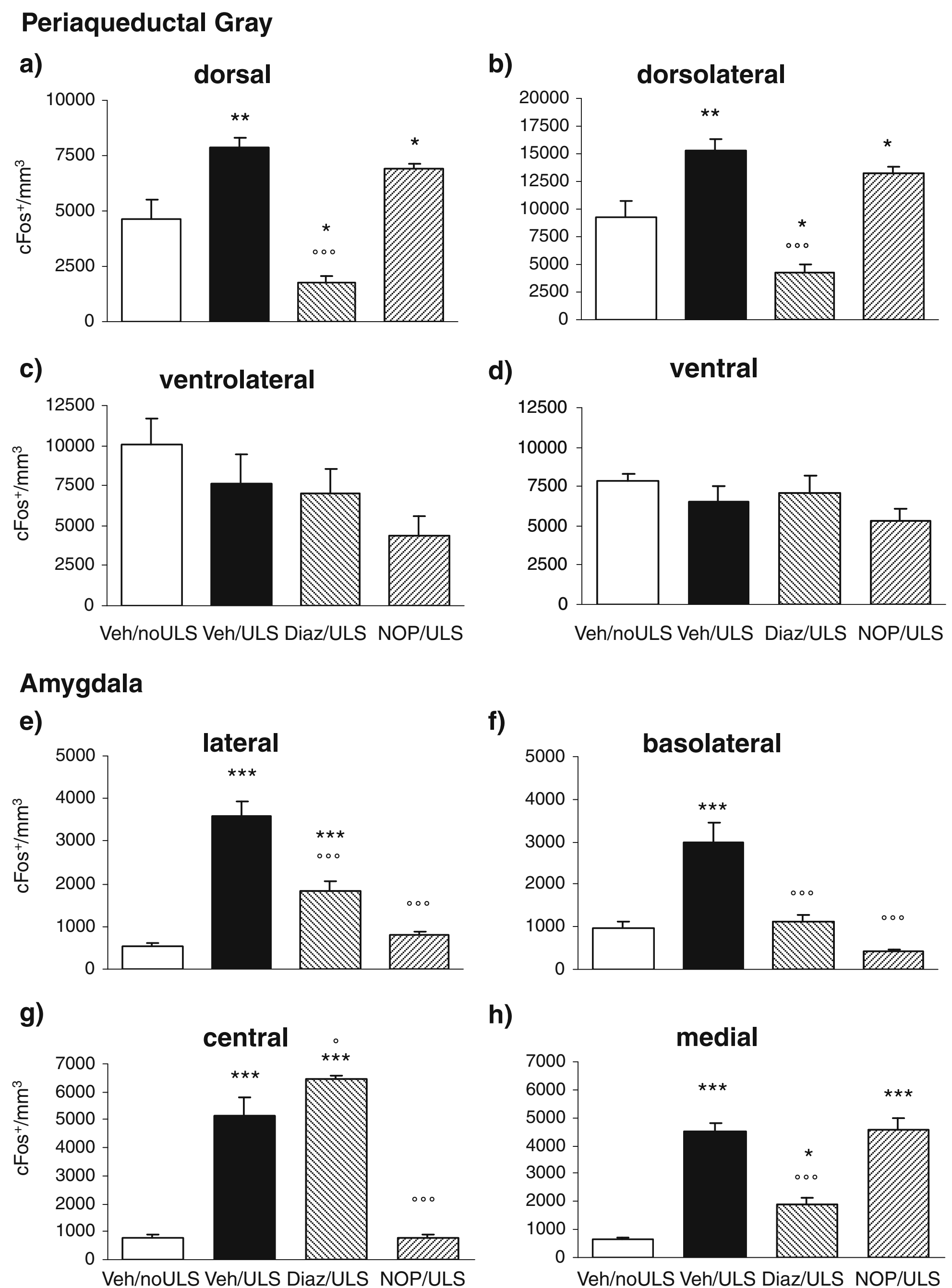

Fig. 3 Number of c-Fos immunoreactive cells per cubic millimeter (mean + SEM) within the different subregions of the PAG (**a**–**d**) and the amygdala (**e**–**h**) after the application of ultrasound (*ULS*). Animals were pretreated with vehicle (*Veh*, $n=4$), diazepam (*Diaz*, $n=4$)), or the nociceptin OFQ peptide receptor agonist Ro 64-6198 (*NOP*, $n=5$). Non-treated animals that were placed into the test cages without ULS served as controls ($n=5$). *Asterisks* indicate significant differences to the vehicle-treated animals without ULS application, while *circles* indicate significant differences to the vehicle-treated animals with ULS application (one-way ANOVA, followed by Tukey's multiple comparison procedure; *,°$p<0.05$; **,°°$p<0.01$; ***,°°°$p<0.001$)

of c-Fos-positive cells after ultrasound application was significantly lower in diazepam-treated animals when compared to vehicle-treated animals ($p<0.001$; Fig. 3b).

Ventrolateral PAG One-way ANOVA did not reveal any significant differences in the number of c-Fos-positive cells between the different treatment groups with or without ultrasound ($F(3,14)=2.54$, $p=0.098$; Fig. 3c).

Ventral PAG One-way ANOVA did not reveal any significant differences in the number of c-Fos-positive cells between the different treatment groups with or without ultrasound ($F(3,14)=1.79$, $p=0.195$; Fig. 3d).

Lateral amygdala One-way ANOVA revealed significant differences in the number of c-Fos-positive cells after the different treatments with or without ultrasound ($F(3,14)=56.01$, $p<0.001$). Tukey's multiple comparison test revealed a significant increase in the number of c-Fos-positive cells after application of ultrasound in the vehicle and in the diazepam groups (both $p\leq 0.001$), but not in the Ro 64-6198 group. In addition, in animals exposed to ultrasound,

the number of c-Fos-positive cells was significantly lower in diazepam- or Ro 64-6198-treated animals when compared to vehicle-treated animals (both $p<0.001$; Fig. 3e).

Basolateral amygdala One-way ANOVA revealed significant differences in the number of c-Fos-positive cells after the different treatments with or without ultrasound ($F(3,14)=22.62$, $p<0.001$). Tukey's multiple comparison test revealed a significant increase in the number of c-Fos-positive cells after application of ultrasound only in the vehicle group ($p<0.001$). In addition, in animals exposed to ultrasound, the number of c-Fos-positive cells was significantly lower in diazepam- and Ro 64-6198-treated animals when compared to vehicle-treated animals (both $p<0.001$; Fig. 3f)

Central amygdala One-way ANOVA revealed significant differences in the number of c-Fos-positive cells after the different treatments with or without ultrasound ($F(3,14)=101.43$, $p<0.001$). Tukey's multiple comparison test revealed a significant increase in the number of c-Fos-positive cells after application of ultrasound only in vehicle- and diazepam-treated animals (both $p<0.001$). The number of c-Fos-positive cells after ultrasound application was significantly higher in diazepam-treated animals when compared to vehicle-treated animals ($p=0.046$) and significantly lower in Ro 64-6198-treated animals when compared to vehicle- or diazepam-treated animals (both $p<0.001$; Fig. 3g). Therefore, the central amygdala is the only amygdaloid nuclei in which Ro 64-6198, but not diazepam, significantly reduced ultrasound-induced c-Fos expression.

Medial amygdala One-way ANOVA revealed significant differences in the number of c-Fos-positive cells after the different treatments with or without ultrasound ($F(3,14)=48.46$, $p<0.001$). Tukey's multiple comparison test revealed a significant increase in the number of c-Fos-positive cells after application of ultrasound in all treatment groups (Veh $p<0.001$; diazepam $p=0.033$; Ro 64-6198 $p<0.001$). The number of c-Fos-positive cells after ultrasound application was significantly lower in diazepam- but not in Ro 64-6198-treated animals when compared to vehicle-treated animals ($p<0.001$; Fig. 3h).

Discussion

The present study suggests that ultrasound-induced wild running as an animal model for panic disorders fulfills several aspects of face and construct validity. We showed that ultrasound-induced defensive behaviors in LH rats are accompanied by tachycardia, a physiological symptom of human panic attack (DSM IV 2003), and an increase in core body temperature, which also reflects autonomic activation. In addition, we observed that ultrasound application has no effect on plasma corticosterone level, which is also true for cortisol levels in humans following a panic attack (Graeff 2007). Finally, we confirmed the finding of Neophytou et al. (2000) that ultrasound induces c-Fos expression in the dorsal regions of the PAG and the amygdala, two regions frequently associated with human panic disorder (reviewed in Graeff and Del-Ben 2008), and demonstrated that these expressions were differentially modulated by the anxiolytic with panicolytic properties diazepam and the preclinical anxiolytic Ro 64-6198.

The observed increases in heart rate and core body temperature are known autonomic symptoms of human panic attacks (Freedman et al. 1984; Cameron et al. 1987; DSM IV 2003) and animal panic reactions (Gray and McNaughton 2000). However, since tachycardia and hyperthermia are common features of aversive autonomic activation, they are considered as more general autonomic markers of classical "fight and flight" reaction. Accordingly, hyperthermia in rats and mice can be induced by several types of unconditioned aversive stimuli such as handling (Bouwknecht et al. 2000; Olivier et al. 2003), restraint stress (Vidal et al. 1984), exposure to a novel environment (Vidal et al. 1984), or mild electrical foot shock (Pechnick and Morgan 1987). In this respect, hyperthermia has been used as a valuable preclinical model for fear/anxiety in rodents (Zethof et al. 1995; Olivier et al. 2003). Similarly to hyperthermia, stress-induced increase in heart rate can be experimentally provoked by exposing rodents to several experimental stressors such as handling (Bouwknecht et al. 2000), exposure to novelty (Van Den Buuse et al. 2001), or physical stressor (Farah et al. 2004). In addition, previous studies have shown that electrical or chemical stimulation of the dlPAG in rats (Carrive 1993; Bandler et al. 2000; Schenberg et al. 2001) and humans (Iacono and Nashold 1982) can evoke autonomic arousal similar to that observed during exposure to a fearful situation and during panic attacks, respectively. Altogether, this suggests that hyperthermia and tachycardia induced by ultrasound application is an expected defensive autonomic reaction and suggests that the ultrasound-induced defensive behavior test fulfills this element of face validity for panic disorder.

In our second experiment, there were no significant differences in plasma corticosterone levels between animals which were exposed to ultrasound and those that were not. Thus, even though ultrasound application is a highly aversive event for LH rats, it did not increase plasma corticosterone levels, at least under conditions of the current study. This was true for non-habituated animals as well as for animals that underwent a habituation procedure. The values in these control groups, ranging from 60 to

125 ng/ml, are in agreement with reported values in LH rats (Schrijver et al. 2002) and in other strains (Uchida et al. 2008) under experimental conditions. Acute stressors such as restraint stress increase these values to 200–400 ng/ml, indicating that the lack of effect of ultrasound was not due to a ceiling effect. The habituated group showed a somewhat higher baseline corticosterone level than the non-habituated group, suggesting that the repeated exposure to the testing procedure might even have increased plasma corticosterone levels, although some inter-experimental variation cannot be excluded (i.e., these two experiments were not performed on the same day or in the same period). This is not entirely surprising as daily injections of saline for 14 days in LH rats resulted in plasma corticosterone levels of more than 200 ng/ml (AlAhmed and Herbert 2008), suggesting that they do not readily habituate to mild stressors. Be that as it may, our findings clearly indicate that ultrasound did not produce an increase in corticosterone in two independent experiments. As mentioned in the "Introduction," absence of an increase in cortisol is a key finding in human panic attacks, implying that the ultrasound-induced defensive behavior test fulfills also this element of face validity for panic disorder. Similar findings were reported in the rat dPAG stimulation model (summarized in Schenberg et al. 2001; Graeff et al. 2005; Graeff 2007).

In our last experiment, we confirmed the finding of a previous study (Neophytou et al. 2000) that ultrasound exposure increases the number of c-Fos immunoreactive cells selectively in the dPAG and dlPAG, as well as in several amygdaloid subregions, i.e., brain regions strongly associated with human panic attacks (Garakani et al. 2007; Graeff and Del-Ben 2008; Schenberg et al. 2001; Javanmard et al. 1999). In addition, we observed that increases in c-Fos expression in the dPAG and the dlPAG could be prevented by pretreatment with the clinically effective anxiolytic/panicolytic diazepam at a dose previously shown to significantly and selectivity reduce ultrasound-induced escape behaviors (Nicolas et al. 2007). In contrast, Ro 64-6198, at a dose which significantly and selectively reduced ultrasound-induced freezing, but not escape behavior (Nicolas et al. 2007), had no effect on c-Fos expression within the PAG. Other anxiolytics without panicolytic efficiency, like buspirone, were not tested in this experiment because our former experiments have shown that buspirone had no effect on the investigated behaviors, neither on escape nor on freezing (Nicolas et al. 2007).

These findings are in line with other preclinical studies which used artificial electrical or chemical stimulation of the dorsal PAG regions. In these studies, benzodiazepines, when injected systemically or directly into the d/dlPAG, effectively increased the stimulation threshold for d/dlPAG stimulation-induced escape behaviors (Brandão et al 1982; Graeff et al. 1986; Bovier et al. 1982; Jenck et al. 1995), while Ro 64-6198 did not (Jenck et al. 2000). Notably, Neophytou et al. (2000) have shown that ultrasound exposure increases c-Fos immunoreactivity within the dPAG/dlPAG in LH rats, but not in Wistar rats, which also did not show any escape response. Taken together, this suggests a correlation between increased c-Fos immunoreactivity in the dorsal PAG regions and the escape response, which is in line with the proposed key role for the dorsal PAG regions in panic attacks (reviewed in Graeff 2004; Graeff and Del-Ben 2008).

In the amygdala, pretreatment with diazepam attenuated the ultrasound-induced increases of c-Fos immunoreactivity within the medial, the basolateral, and the lateral, but not the central subregion. Pretreatment with Ro 64-6198 effectively prevented the ultrasound-induced increases in c-Fos immunoreactivity in all but the medial subregions. Together with the finding that inactivation of the medial amygdala has been reported to increase the escape threshold for dPAG stimulation and inhibited escape behavior in the elevated T-maze (Herdade et al. 2006), these results support the hypothesis that the medial amygdala may play a role in modulation of dPAG-induced escape behavior. However, since the medial amygdala shares no direct connections with the PAG (Rizvi et al. 1991; Vianna and Brandão 2003), the neural substrate of this potential functional interaction remains to be elucidated.

In the central amygdala, ultrasound-induced increases in c-Fos expression could be effectively prevented by pre-treatment with Ro 64-6198, but not with diazepam, which, on the contrary, produced a significant increase. The latter results are supported by former studies which investigated the effects of the treatment with diazepam on c-Fos expression in the amygdala of stressed rats, also reporting increased c-Fos expression in the central amygdala by diazepam (Beck and Fibiger 1995; de Medeiros et al. 2005). Additionally, another study showed that diazepam increased c-Fos expression also in the central amygdala of habituated rats without any behavioral task (Salminen et al. 1996). The reduced c-Fos expression in the central amygdala after pretreatment with Ro 64-6198 could thus be related to its inhibition of ultrasound-induced freezing (Nicolas et al. 2007). This hypothesis is supported by d/dlPAG stimulation studies which revealed that lesions of the central amygdala reduced post-stimulation freezing (Martinez et al. 2006; Macedo et al. 2005) and by a study showing that lesions of the central amygdala impaired the expression of freezing in a classical fear conditioning test (Sullivan et al. 2004). Somewhat confusing is that the central amygdala is the only amygdaloid nucleus sharing direct and reciprocal connections with the PAG (Rizvi et al. 1991; da Costa Gomez and Behbehani 1995; Vianna and Brandão 2003). Further studies are therefore needed to clarify the exact interactions between the central amygdala, the PAG, and the animal's behavior under these specific conditions.

In the lateral and the basolateral amygdala, both diazepam and Ro 64-6198 reduced the ultrasound-induced increase in c-Fos immunoreactivity. Since both drugs show differential behavioral effects, this might suggest that the lateral and basolateral amygdala are not specifically involved in the expression of the differential defensive behaviors observed after stimulation of the d/dlPAG regions. Additionally, other studies have shown that inactivation of the lateral and the basolateral amygdala did not change the d/dlPAG stimulation threshold for freezing or escape response, but did reduce contextual freezing when animals were re-exposed to an arena where they had been stimulated in the d/dlPAG regions before (Martinez et al 2006). Martinez et al. (2006) therefore hypothesized that the lateral and basolateral amygdala do not contribute to the innate response to unconditioned stimuli, but may rather contribute to the later processing of the threatening stimuli allowing the association between the unconditioned and the conditioned stimuli. To further test this hypothesis, it could be of interest to examine the effect of re-exposure to the test arena following ultrasound-induced escape responses in LH rats.

One limitation of the current study is that we did not study the effects of diazepam and Ro 64-6198 on c-Fos expression in the absence of ultrasound application to understand the specificity of their effects in each of the brain areas, and it would be of interest in future studies to include such groups. Another limitation is that we used Ro 64-6198 as an anxiolytic without panicolytic activity, even though there are no clinical data for this compound. We chose Ro 64-6198 because we wanted to compare two compounds with specific effects on escape and freezing, whereas buspirone had no specific effects on freezing in our model (Nicolas et al. 2007). Nevertheless, testing a clinically validated compound such as buspirone would be important to confirm the present findings.

Altogether, the differential modulation of c-Fos expression by diazepam and Ro 64-6198 seems to reflect their differential modulation of behaviors and may therefore help to delineate the neuronal structures underlying the different behavioral responses to d/dlPAG stimulation by ultrasound. In short, the ultrasound-induced wild running, which may be a correlate of relevance to human panic reactions (e.g., Schenberg et al. 2001), seems to be mediated by brain regions in which diazepam specifically attenuated c-Fos expression, namely, the d/dlPAG and the medial amygdala. On the other hand, freezing may be modulated by brain regions in which Ro 64-6198 attenuated c-Fos expression, namely, the basolateral and lateral amygdala and more specifically the central amygdala. In this way, the current and future studies utilizing ultrasound-induced defensive reactions may also help to further differentiate the neural correlates of panic and anxiety.

In conclusion, the present study suggests that ultrasound-induced wild running is an animal model for panic disorders that fulfills several aspects of face and construct validity. A significant advantage of this model is that it uses a noninvasive stimulus which needs neither time-consuming training nor surgery, making it an attractive animal model of human panic attacks. Furthermore, it overcomes the main criticism of the artificial PAG stimulation model (reviewed in Del-Ben and Graeff 2009) in that it shows more than a simple PAG-induced panic reaction, but also a broad activation of neuronal networks that participate in the regulation of fear and anxiety. More definite (predictive) validation of the model should include the examination of chronic treatment with panicolytic drugs like selective serotonin reuptake inhibitors.

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