GLUTAMATE RECEPTOR ANTAGONISM IN INFERIOR COLLICULUS ATTENUATES ELEVATED STARTLE RESPONSE OF HIGH ANXIETY DIAZEPAM-WITHDRAWN RATS

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Abstract—Rats segregated according to low (LA) or high (HA) anxiety levels have been used as an important tool in the study of fear and anxiety. Since the efficacy of an anxiolytic compound is a function of the animal’s basal anxiety level, it is possible that chronic treatment with a benzodiazepine (Bzp) affects LA and HA animals differently. Based on these assumptions, this study aimed to provide some additional information on the influence of acute, chronic (18 days) and withdrawal effects (48 h) from diazepam (10 mg/kg), in rats with LA or HA levels, on startle response amplitude. For this purpose, the elevated plus-maze (EPM) test was used. In addition, the role of glutamate receptors of the central nucleus of the inferior colliculus (cIC), the most important mesencephalic tectum integrative structure of the auditory pathways and a brain region that is linked to the processing of auditory information of aversive nature, was also evaluated. Our results showed that, contrary to the results obtained in LA rats, long-term treatment with diazepam promoted anxiolytic and aversive effects in HA animals that were tested under chronic effects or withdrawal from this drug, respectively. In addition, since Bzp withdrawal may function as an unconditioned stressor, the negative affective states observed in HA rats could be a by-product of GABA-glutamate imbalance in brain systems that modulate unconditioned fear and anxiety behaviors, since the blockade of alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) and N-methyl-D-aspartate (NMDA) glutamate receptors in the cIC clearly reduced the aversion promoted by diazepam withdrawal. © 2009 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: diazepam withdrawal, anxiety, elevated-plus-maze, inferior colliculus, startle response, glutamate.

In general, anxiety can be classified depending on its nature. In this case, two primary levels appear, called state or trait anxiety. The first is related to that type of anxiety experienced under certain conditions, which is also accepted as a “normal” state, and is elicited in response to anxiety-provoking situations. The second is related to “pathological” conditions and is characterized chiefly by an innate individual predisposition to respond, accompanied by the appearance of powerful feelings of distress in response to the presence of innocuous or weak anxiety stimuli (Spielberger, 1966; Endler and Kocovski, 2001).

With regard to the point above, rats selectively bred for their anxiety-related behavior have been used as an important tool in the neurobiological study of anxiety- and fear-related phenomena (Liebsch et al., 1998a,b; Landgraf and Wigger, 2002; Salome et al., 2004). In order to demonstrate the differentiation in the behavioral performance that characterizes low- (LA) and high-anxiety (HA) rats, the elevated plus-maze (EPM) has primarily been used as the recommended anxiety test. With respect to this animal model of unconditioned anxiety, it has been demonstrated that HA rats reveal a marked and robust elevation in their emotionality when submitted to this apparatus, when the time spent in the open arms is the foremost measure (Liebsch et al., 1998b; Henniger et al., 2000; Landgraf and Wigger, 2002; Salome et al., 2004).

In addition to its peculiar somatic signs, withdrawal from most drugs of abuse, including alcohol, psychostimulants, nicotine, opiates and benzodiazepines (Bzp), can promote similar disturbances in affective states (Markou et al., 1998). With respect to Bzp it is well established that, as in humans, the ability of this class of anxiolytic drugs to promote its effects is a function of the laboratory animal’s basal anxiety levels (Green, 1991; Lader, 1991; Griebel, 1995; Rodgers et al., 1997). In this case, it is possible that long-term treatment with diazepam affects LA and HA rats in a different way, so that distinctive patterns of symptoms are achieved when the animals are tested under acute and chronic effects or when withdrawn from this drug.

It has been demonstrated that, after abrupt discontinuation of chronic Bzp exposure, patients can experience a number of symptoms indicative of a dependent state, extreme anxiety levels being the earliest to arise and the most persistent signal of withdrawal (Marks, 1978; O’Brien, 2005). In laboratory animals increased levels of acoustic startle-induced 22-kHz ultrasonic vocalization (USV) and enhanced startle response amplitude to an auditory startling-eliciting stimulus were observed (Vivian et al., 1994; Martijena et al., 1996; De Ross et al., 2008).

Anxiety is a complex and multifactorial emotional state whose primary characteristics are linked to the appearance of longstanding heightened autonomic behavioral arousal associated with a concurrent sustained increase in avoidance behavior (Lowry et al., 2005).

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Abbreviations: AEP, auditory evoked potential; AMPA, alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate; AP-7, 2-amino-7-phosphono-heptaneate; Bzp, benzodiazepine; cIC, central nucleus of the inferior colliculus; dPAG, dorsal periaqueductal grey; EPM, elevated plus-maze; GDEE, glutamic-acid diethyl ester; HA, high anxiety; IC, inferior colliculus; LA, low anxiety; NMDA, N-methyl-D-aspartate; SPL, sound pressure level; USV, ultrasonic vocalization.
Startle is a stereotyped response consequent to the presentation of a sudden and unexpected stimulus. In most of the cases the stimulus is acoustic, but other modalities such as tactile, visual or vestibular are also used (Grillon, 2007). As experienced by mammals, the response is composed of motor, autonomic and emotional components that enable the flight reaction and/or protection of the body from sudden attack (Meinck, 2006; Grillon, 2007). In both humans and laboratory animals, fear and anxiety-inducing stimuli heighten the sense of awareness, resulting in being more easily startled (Grillon, 2007). Most important however, this reflex is magnified in patients with anxiety disorders (Grillon et al., 1998).

The study of Fontanesi et al. (2007) showed that rats withdrawn from diazepam have decreased both time spent and number of entries in the open arms of the EPM. This increase in anxiety measures was accompanied by enhanced Fos-immunolabeling in brainstem structures mainly linked to the modulation/expression of unconditioned anxiety- and fear-related behaviors, such as the dorsal peri-aqueductal grey (dpAG) and the inferior colliculus (IC). In line with this, results from the study of Souza-Pinto et al. (2007) demonstrated that the increased unconditioned anxiety promoted by diazepam withdrawal could be counteracted by local microinjections into the dpAG of glutamate antagonists, suggesting that the expression of this negative affective state could be due, in part, to the enhancement of excitatory mechanisms (compensatory nervous system action) that develops, probably in response to diazepam-induced chronic enhancement of GABAergic inhibition (Stephens, 1995).

The involvement of the IC on fear and anxiety states has been suggested (Graeff, 1990; Brandão et al., 1993, 2001). The study of Baas et al. (2006) showed that brainstem auditory evoked potentials (AEp), represented by wave V, in which the main generator is the IC, are increased during experimentally induced anxiety in healthy volunteers. Moreover, blockade of the glutamate receptors of the central nucleus of the inferior colliculus (cIC) of this midbrain region is well-known for its anxiolytic-like action, and is frequently observed in naive rats pre-treated with glutamate antagonists and then submitted to anxiety-provoking stimuli (Brandão et al., 1999, 2005).

Based on these assumptions, the aim of this study was to provide additional information on the acute effects and withdrawal from long-term treatment with the prototypic Bzp diazepam on the anxiety-like behavior of rats that were previously selected according to their anxiety level (LA or HA), as revealed by the EPM test. For this purpose, the analysis of startle response amplitude was taken as a measure of unconditioned anxiety in both LA and HA rats. The role of glutamate neurotransmission in the cIC on the modulation of the acoustic startle was also evaluated, in both classes of animal. Since this midbrain region is over-active in diazepam withdrawn rats, as revealed by the Fos-protein immunolabeling technique (Fontanesi et al., 2007), we hypothesized that enhanced glutamatergic neurotransmission in the cIC could underlie the unconditioned anxiety-like behavior elicited by auditory stimuli in rats tested under diazepam withdrawal, in the same way that it does in naive rats made experimentally anxious.

EXPERIMENTAL PROCEDURES

Animals

One hundred sixty-four male Wistar rats, weighing 100–110 g at the beginning of the treatment (except for experiment II) were used. The rats were purchased from the animal house of the University of São Paulo (Ribeirão Preto, São Paulo, Brazil). They were housed in groups of four in Plexiglas-walled cages, lined with wood shavings that were changed every 3 days, and were maintained in a 12-h light/dark cycle (lights on 07:00 h) at 24±1°C. The rats were given free access to food and partial access to water. Before the beginning of the treatments, the animals had a 3-day habituation period to the lodging conditions, deprivation, and drinking procedures. Except for experiments II and IV (n = 64), in which startle measurement (II) was later challenged with glutamate antagonists (IV), independent groups of animals were used, as follows: Experiment I: n = 20; Experiment III: n = 40; Experiment V: n = 40. A rigorous approach was taken on issues such as animal welfare and accommodation. In addition, efforts were made to reduce the number of animals as well as refining the procedures used in order to minimize suffering. The experiments reported in this article received local Ethics Committee approval, being performed in compliance with the recommendations of Brazilian Society for Neuroscience and Behaviour which, in turn, are in accordance with the rules of the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Administration of diazepam by oral route

The majority of studies in the literature that investigate the chronic effects of a drug of abuse have mainly used i.p. injections as the method of delivery of the drug. However, it was demonstrated that rats subjected to chronic systemic injections of placebo solutions tend to present an increase in anxiety levels when tested in the elevated-plus maze (Griebel et al., 1994). To circumvent this problem, we used a new model of oral drug intake, adapted by Schleimer et al. (2005), in which the animals voluntarily drink the drug or control solutions. However, the self-administration of diazepam p.o. in rats suffers the bias of gustative and temporal factors and a delay in the production of its reinforcing effects. In this case, water deprivation and sucrose were added to our experimental design to help circumvent this problem. In fact, the use of deprivation for the establishment of motivational drives has been very successful when emotional aspects of behavior are evaluated. On the other hand, it is well known that sucrose, by itself, has high reinforcing effects, and it has been found that sucrose-withdrawn animals have increased anxiety levels. With regard to this point, we have previously shown that the maximum dose of 100 μg of sucrose does not have any effect on the motivational drive of the animals as neither the percentage of entries nor the time spent in the open arms of the plus maze was changed in these animals, in comparison with the no-treatment group (Fontanesi et al., 2007).

The procedure used for diazepam oral intake has been described previously (Fontanesi et al., 2007). Briefly, the animals were submitted on a daily basis to 14 h of water deprivation (19:00 to 09:00 h), followed by 10 h of water ad libitum (09:00 to 19:00 h). This procedure began 3 days before the start of the 18-day treatment, as an acclimatization period for the animals to the drinking procedure. Diazepam was separately dissolved at a concentration of 10 mg/ml of tap water plus propylenoglycol (5%). This water–propylenoglycol–diazepam mix was offered once daily in a volume of 1 ml/kg diluted in a solution of 2 ml of tap water added to sucrose (5%). The water–sucrose–propylenoglycol mix was also used as a control solution. The solutions were made available to the animals in glass pipettes of 5 ml in a procedure...
that was developed with the express purpose of making all animals receive the same dose and volume of vehicle or drug. At the end of the deprivation period (09:00 h), the animals (four per cage) were isolated in their own cages through the use of a Plexiglas cross-division. At this point, the glass pipettes containing the solutions described above were offered to the animals. In this condition, each animal received an equal volume of the vehicle solution (2 ml of tap water + sucrose (5%) + 5% of propylene glycol) or diazepam solution (2 ml of vehicle solution + diazepam 10 mg/kg) daily. At the end of vehicle or drug intake, the Plexiglas cross-divisions were removed and water was made available. Except for the first 2 days of treatment, in which control and experimental solutions were available for 30 min, those animals that did not drink the sucrose or diazepam solutions for 10 min were removed from the experiments. This was necessary to avoid prolonging the deprivation period. Experimental sessions were conducted for 30 min (termed the chronic effects condition, in which the animals were tested during the effect of the drug) or 48 h after the last oral drug intake (the withdrawal condition, in which the animals were tested free of the drug).

**Experiment I: Effects of acute diazepam oral intake on the startle response of rats selected for their LA or HA levels**

In this experiment 20 adult male Wistar rats, weighing 280–300 g, were used. Initially, the rats were separated according to their propensity to display HA or LA anxiety-like behaviors when submitted to a procedure that commonly evokes such an emotional response, the EPM (third day of the 3-day habituation period). To this end, a biased version of the traditional plus-maze was used (Salome et al., 2004). Precisely 24 h after the initial selection in the plus-maze, the experiments on the startle response were initiated. As the rats used in this study differed in age (and weight) from those used in the remaining experiments, a separate statistical analysis of the data obtained in the EPM was performed.

**Experimental apparatus.** Plus-maze. The EPM was constructed from dark plywood and had two open arms (50×10 cm), perpendicular to two closed arms of equal dimensions, surrounded by 40-cm walls. The apparatus was elevated 50 cm from the floor (Anseloni and Brandão, 1997). For our purposes, access to the closed arms was prevented through the use of a closed arm door. In fact, exposure to the open arm of the EPM as a reliable anxiety test has been validated in a well-designed study by Salomé et al. (2006). In this study, statistical reports strongly confirmed that the main distinction between HA and LA rats was primarily based on anxiety since, concerning the general locomotor activity, HA and LA animals did not present any significant difference on this measure, as the distance travelled was similar during the 5-min test. In addition, as revealed by discriminant analysis, the high-test probability to differentiate HA from LA rats, in this test, was linked to only one parameter, closely related to trait anxiety: the time spent in the distal zone of the open arms. This later parameter was, indeed, the anxiety index used in our study.

The open arms were divided into equal main sections, referred to as the proximal part (the half adjacent to the center) and the distal part (the half near the edge of the open arms). A 1-cm wooden rim surrounded the open arms in order to prevent falls from the plus-maze. The apparatus was located inside a refrigerated room with constant noise (50 dB). Behavior in the EPM was documented by a camera (Everfocus, Duarte, CA, USA) linked to a monitor and video-cassette. This device, located outside the experimental room, allowed the recordings to be analyzed later. Luminosity at the level of the open arms of the maze was 60 lx.

**Startle response.** Two sets of startle response apparatus were used. Individually, they consisted of a wire-mesh cage (16.5×5.1×7.6 cm) that was attached to a stabilimeter (response platform – 36.5×11.5×4.5 cm) with four thumb-nail-screws, suspended within a ventilated plywood sound attenuation box (96×48×45 cm), divided by two chambers (48×48×45 cm) in which the startle hardware was localized. The floor of the wire-mesh cage consisted of six stainless steel bars 3.0 mm in diameter and spaced 1.5 cm apart. The startle amplitude was recorded within a time window of 200 ms after the onset of the startle stimulus. A loudspeaker located 15 cm from the rear of each chamber was used to deliver the startle stimuli, in addition to continuous background noise (white noise, 60 dB) delivered during the entire session. The rats’ startle reaction generated pressure on the startle meter and analog signals were amplified, digitized and analyzed by the startle measurement system software (Insight, São Paulo, Brazil), which also controlled all parameters of the session (intensity of the acoustic stimulus, inter-stimulus interval, etc.). In order to ensure equivalent sensitivities of the response platforms over the test period, calibration procedures were conducted prior to experimentation. Animal behavior was recorded by an infrared camera (Safety View, São Paulo, Brazil) located behind the stabilimeter, with the signal being relayed to a video and a monitor in another room via a closed circuit system.

**Experimental procedure.** Plus-maze. The tests on EPM began after a 3-day habituation period to the lodging conditions. For each group of four animals that were housed together in the same box in the animal house during the 3-day adaptation period, a procedure was established to divide the animals into pairs: those that spent more time in the distal parts of the open arms of the plus-maze were allocated to the LA group, and the remaining two animals were allocated to the HA group. Each rat was placed at the center of the EPM facing a closed arm door and was allowed to explore the environment for 5 min. The measures taken were the percentage of entries and time spent in the center and the proximal and distal parts of the open arms. Each animal was tested only once. The apparatus was cleaned with 20% ethanol and water before each test. After exposure to the EPM, the animals were allocated to one of the two groups (LA or HA behavior) and maintained in this condition throughout all experiments.

**Startle response.** The first startle session was conducted 30 min after oral intake of sucrose in both LA and HA groups, 24 h after the EPM sessions. After an additional 24 h, the same procedure was repeated; however, this time the animals were tested 30 min after oral intake of diazepam (10 mg/kg). Overall, two groups were formed in a within-subjects design: LA×sucrose and diazepam oral intake; HA×sucrose and diazepam oral intake. These experiments were conducted in a simple paradigm routinely used in our laboratory ( Nunes Mamede Rosa et al., 2005; Cabral et al., 2006). The acoustic startle test session consisted of two parts. The first was a 5-min period of acclimatization to the startle test chamber. Except for the background noise, no acoustic startle stimulus was presented during this period. The second part consisted of 40 presentations of an acoustic startle stimulus (pulse, 110–120 dB, 50-ms bursts of white noise having a rise–decay time of 5 ms). The inter-stimulus interval of 30 s used between trials was based on previous studies performed in our laboratory ( Nunes Mamede Rosa et al., 2005; Cabral et al., 2006). The testing session lasted for 20 min. Data obtained were first stored on a hard disk and then transferred to tables in a spreadsheet program for off-line analysis.

**Statistical analysis.** In order to demonstrate that LA and HA differ significantly in terms of the results obtained in the EPM test, a one-way ANOVA was used (data not shown). Startle responses were averaged for each animal across the entire session and, for each raw data point, the square root was calculated and used for statistical purposes. After that, a two-way repeated measures ANOVA was performed. The treatments (sucrose×diazepam) and the anxiety levels (LA×HA) were used as the main factors, the treatments being the repeated variable. The results obtained were presented as mean±SEM. Statistical analyses were followed when appropriate (P<0.05) by the Fisher LSD post hoc test.
Results. Fig. 1 shows the influence of acute diazepam oral intake on the startle response evoked by LA and HA rats. Two-way repeated measures ANOVA showed significant differences between treatments (sucrose × diazepam) \( F_{1,18} = 5.40; P < 0.05 \). The post hoc Fisher-LSD test revealed that this difference could be attributed to the effect of diazepam, which caused a decrease in the amplitude of the startle response in LA rats, when compared with their respective control. No statistically significant effect on anxiety levels (LA × HA) \( F_{1,18} = 1.53; P > 0.05 \) was observed. ANOVA also showed no significant interaction between treatments and anxiety levels \( F_{1,18} = 2.32; P > 0.05 \).

Experiment II: Effects of chronic intake of diazepam and withdrawal on the startle response of rats selected for their LA or HA levels

Experiments were performed using eighty male Wistar rats, weighing 110–120 g (postnatal day 30). During treatment, four animals were excluded, as a result of problems during surgery (cannula clogging, prosthesis infection, or death). Another 12 animals were removed because the time that they spent in the center or distal parts of the maze was either below 10% or above 90% of total test time. In order to separate animals according to their basal anxiety levels, the same procedure described in experiment I was used. Drug treatment began 24 h after the EPM tests (day 1 of the 18-day chronic treatment period).

Experimental apparatus. Plus-maze. This was as described in experiment I.

Startle response. This was as described in experiment I.

Experimental procedure. Plus-maze. This was as described in experiment I.

Startle response. For the startle response experiment, the procedure used was the same as that used in experiment I, except that the animals were tested after 18 days of chronic treatment with sucrose or diazepam. Four groups were formed (n = 16 for each group): sucrose LA, sucrose HA, diazepam LA and diazepam HA. The same animal was tested 30 min (under chronic drug effects) and 48 h after the last sucrose or diazepam intake (under withdrawal effects).

Statistical analysis. EPM analysis was performed in the same way as described in experiment I (data not shown). The results from the startle reflex test are reported as mean ± SEM. Similar to the procedure used in experiment I, startle responses were averaged for each animal across the entire session and, for each raw data point, the square root was calculated and used for statistical purposes. After that, a three-way repeated measure ANOVA test was performed with treatments (sucrose × diazepam), anxiety levels (LA × HA) and condition during the test (under drug effects × withdrawal) as the main factors. Condition was the repeated variable. Statistical analyses were followed, when appropriate \( (P < 0.05) \), by the Fisher LSD post hoc test.

Results. Fig. 2 shows the influence of the chronic effects of and withdrawal from diazepam on the startle response of LA and HA rats. Three-way repeated measures ANOVA showed marginal effects for the treatment (sucrose × diazepam) \( F_{1,60} = 3.86; P = 0.054 \), a significant difference between the levels of anxiety (LA × HA) \( F_{1,60} = 5.43; P < 0.05 \), the absence of interaction between treatment × levels of anxiety \( F_{1,60} = 0.003; P > 0.05 \), no significant effects for condition (chronic × withdrawal) \( F_{1,60} = 1.44; P > 0.05 \), a significant interaction between condition × treatments \( F_{1,60} = 5.31; P < 0.05 \) and condition × anxiety levels \( F_{1,60} = 4.45; P < 0.05 \), and no significant interactions among condition × treatments × levels of anxiety \( F_{1,60} = 3.41; P > 0.05 \). The post hoc Fisher LSD test showed that the startle reflex evoked by HA sucrose pre-treated animals was not statistically different from that in LA rats. Similarly, 48 h of sucrose withdrawal did not change the amplitude of the startle response evoked by the same set of previously tested animals. 30 min after the last sucrose oral intake (chronic condition). With respect to the effects of diazepam treatment, LA rats tested under the effects of the drug or during withdrawal did not differ from each other. However, in HA rats, diazepam reduced the startle amplitude. The same animals, when tested during withdrawal, showed a significant increase in this response.

Statistical analysis. EPM analysis was performed in the same way as described in experiment I (data not shown). The results from the startle reflex test are reported as mean ± SEM. Similar to the procedure used in experiment I, startle responses were averaged for each animal across the entire session and, for each raw data point, the square root was calculated and used for statistical purposes. After that, a three-way repeated measure ANOVA test was performed with treatments (sucrose × diazepam), anxiety levels (LA × HA) and condition during the test (under drug effects × withdrawal) as the main factors. Condition was the repeated variable. Statistical analyses were followed, when appropriate \( (P < 0.05) \), by the Fisher LSD post hoc test.

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Statistical analysis. EPM analysis was performed in the same way as described in experiment I (data not shown). The results from the startle reflex test are reported as mean ± SEM.
Experiment III: Effects of the chronic intake of and withdrawal from diazepam on the 22-kHz ultrasound emission induced by startle-eliciting stimuli in LA and HA rats

This experiment was designed in order to verify whether the increase in the amplitude of startle response observed in HA rats withdrawn from diazepam, as shown by experiment II, was due to withdrawal-induced attenuation of the drug’s anxiolytic effects or a response to the negative affective state elicited by withdrawal. For this purpose, 40 naive Wistar rats (four groups, n=10 for each group) were submitted to chronic sucrose or diazepam oral intake and tested in the conditions described above (see experiment II). Twenty-two kHz USV emissions were registered immediately after the end of the startle experiments.

Experimental apparatus. Plus-maze. This was as described in experiment I.

Startle auditory stimuli inducing 22-kHz USV. The startle response was measured as described in experiment II. For recording and analysis of 22-kHz USV (Bassi et al., 2007; Nobre et al., 2003; Nunes Mamede Rosa et al., 2005), one ultrasound bat detector (Ultra Sound Advice, London, UK) was mounted on the rear wall of the startle chamber, linked to a multichannel audio apparatus with 20-kHz low band pass (cutoff) filter (Noldus Instruments, Amsterdam, The Netherlands) and then fed to a computer. The choice of the frequencies was based on previous studies that showed that rats exposed to dangerous situations display an enhancement of the emissions of 22- and 24-kHz USV that do not differ from each other (Nobre and Brandão, 2004; Nunes Mamede Rosa et al., 2005).

Experimental procedure. Plus-maze. This was as experiment I.

Startle auditory stimuli inducing 22-kHz USV. For the USV measurements, a bat detector was tuned to a 22-kHz frequency and the number of calls emitted during the 5 min immediately after the end of the startle sessions was recorded. The number of 22-kHz calls was analyzed by a sonograph (Ultravox signal analysis workstation, Noldus Instruments, The Netherlands), stored on a hard disk, and subsequently transferred to tables in a spreadsheet program (Excel; Microsoft Corp., Mountain View, CA, USA) for off-line analysis. The microphone was calibrated before each session with the use of an audio generator (GW, 1 MHz, NJ, USA).

Statistical analysis. All results from USV were reported as mean±SEM. USV was averaged for each animal across the entire session. For each raw data point, the square root was calculated and used for statistical analysis. Mean 22-kHz emissions were analyzed with a three-way repeated measures ANOVA with treatment (sucrose × diazepam) and anxiety level (LA × HA) as the between-subject factors, and condition (chronic × withdrawal) as the within-subject repeated factor. When P<0.05 all statistical analyses were followed by the Fisher LSD post hoc test.

Results. Statistical analysis revealed no statistically significant effect of treatment [F(1,36)=1.32, P>0.05], a significant effect of the anxiety level [F(1,36)=26.33, P<0.0001], no significant interactions between treatment × anxiety level [F(1,36)=2.31, P>0.05], a significant effect for condition [F(1,36)=23.72, P<0.0001], and significant interactions between condition × treatment [F(1,36)=39.74, P<0.0001], condition × level of anxiety [F(1,36)=30.40; P<0.0001] and among condition × treatment × anxiety level [F(1,36)=35.06; P<0.0001]. Post-hoc Fisher LSD demonstrated that LA and HA sucrose pre-treated rats did not differ from each other in 22-kHz USV emissions when tested under both chronic and withdrawal conditions (Fig. 3). Diazepam did not cause statistically significant differences in USV emission rates evoked by LA rats, when compared with LA-sucrose rats. In addition, the condition in which these animals were tested also showed no influence on USV rates. On the other hand, HA diazepam pre-treated animals showed a different pattern of 22-kHz USV emissions. In fact, HA rats tested under the effects of diazepam showed a decrease in USV rates compared with those observed in HA sucrose pre-treated rats and with the measurements obtained from the same animals tested under withdrawal. In this condition, HA diazepam-withdrawn rats made more 22-kHz USV calls when compared with both the LA diazepam and the HA sucrose withdrawal pre-treated rats.

Experiment IV: Effects of the blockade of the glutamate receptors of the cIC on the amplitude of the startle response of LA and HA diazepam-withdrawn rats

This experiment was performed in order to study the effects of the blockade of the glutamate receptors of the cIC on the amplitude of the startle response induced by auditory stimuli, in LA and HA rats, tested under the effects of diazepam or drug withdrawal.

Experimental apparatus. Startle response platform was as described in experiment II.

Experimental procedure. After collecting the startle measurements during withdrawal (experiment II), each group formed was subdivided and challenged 2 h after withdrawal sessions with local cIC microinjections of physiological saline or the glutamate antagonists 2-amino-7-phosphonoheptanoate (AP-7) or glutamic acid diethyl ester (GDEE). Five or 10 minutes after the microinjections of GDEE or AP-7, respectively, the animals were placed in the startle apparatus and the behavioral measurements were repeated. The groups formed were sucrose LA×AP-7, sucrose LA×GDEE, sucrose HA×AP-7, diazepam LA×GDEE, diazepam LA×AP-7, diazepam HA×GDEE and diazepam HA×AP-7 (n=8 for each group). The measurements were retaken in the same way as described in experiment II.

Surgery. Surgery was performed on day 14 (4 days before the end of treatment) since long post-surgery periods may cause problems such as loose prosthesis, infections, etc. The animals were anesthetized with tribromoethanol (250 mg/kg i.p.) and mounted in a digital stereotaxic frame (Insight, São Paulo, Brazil). A cannula made from a stainless steel needle (24 G) was directed in the cIC. The upper incisor bar was set at 2.5 mm below the interaural line, such that the skull was placed horizontally between
the bregma and lambda. The cannula was introduced vertically using the following coordinates with the bregma serving as the reference for each plane: AP −8.5 mm; ML 1.5 mm; and DV −2.0 mm (Paxinos and Watson, 2005). The cannula was fixed to the skull by means of acrylic resin and three stainless steel screws. At the end of surgery, each animal received an i.m. injection of a veterinary pentabiotic (120,000 UI, 0.2 ml) followed by an injection of the anti-inflammatory and analgesic banamine (flunixin meglumine, 2.5 mg/kg).

Drugs. GDEE (160 nmol/0.2 μl, Sigma, St. Louis, MO, USA) and AP-7 (10 nmol/0.2 μl, Sigma, St. Louis, MO, USA) dissolved in physiological saline shortly before intracollicular microinjections were used. The vehicle was also used as a control solution. GDEE and AP-7 were microinjected 10 and 5 min before the test, respectively. The doses of the antagonists were selected on the basis of previous studies (Nobre et al., 2004; Brandão et al., 2005).

Microinjection procedures. Two hours after testing (48-h withdrawal), the animals were gently wrapped in a cloth, were hand-held, and a thin dental needle (OD 0.3 mm) was introduced through the guide-cannula until its lower end was 3 mm below its tip. The injection needle was linked to a 5-μl syringe pump (Insight) by means of a polyethylene tube (PE-10) and a volume of 0.2 μl of physiological saline, GDEE or AP-7 was injected over 60 s. The displacement of an air bubble inside the polyethylene tube was used to monitor the microinjection.

Histology. Upon completion of the experiments, the animals were deeply anesthetized with urethane and perfused intracardially with 0.9% physiological saline followed by formalin solution (4%). Three hours later, the brains were immersed in a sucrose solution (30%). After an additional 7 days’ exposure, the brains were frozen. Serial 60-μm brain sections were cut using a cryostat (Leica, Wetzlar, Germany) and stained with Neutral Red in order to localize the positions of the microinjection sites according to Paxinos and Watson’s atlas (2005).

Statistical analysis. Raw data from the startle test were averaged for each animal across the entire session, normalized through the use of square roots, and presented as mean ± SEM. The effects of the glutamate antagonists GDEE or AP-7 on startle reflex of LA or HA rats were analyzed separately using the same procedure described in experiment II. All statistical analyses were followed, when appropriate, by the Fisher LSD post hoc test. *P < 0.05 was considered significant.

Results. Fig. 4 shows a photomicrograph of a representative site and the location of points of microinjections into the cIC. The effects of the AMPA-kainate antagonist GDEE and of the NMDA receptor’s antagonist AP-7 on the magnitude of the startle reflex are illustrated in Fig. 5 (top and bottom, respectively). For the GDEE groups, three-way repeated measures ANOVA showed no significant difference between treatments (sucrose × diazepam) \(F_{1,28} = 1.99; \ P = 0.05\), anxiety levels (LA × HA) \(F_{1,28} = 0.29; \ P = 0.05\) or the main interaction between treatment and anxiety level \(F_{1,28} = 1.83; \ P = 0.05\). A significant effect was detected for condition (withdrawal × GDEE effects) \(F_{1,28} = 57.75; \ P < 0.0001\) and a significant interaction was found between condition × treatment × anxiety level \(F_{1,28} = 4.76; \ P = 0.05\). No statistically significant interaction was found for condition × anxiety level \(F_{1,28} = 0.36; \ P > 0.05\) or among condition × treatment × anxiety level \(F_{1,28} = 0.19; \ P > 0.05\). Post hoc Fisher tests showed that the antagonism of AMPA receptors in cIC with GDEE significantly decreased the startle response in both sucrose and diazepam pre-treated rats independent of their level of anxiety (LA or HA). A very similar pattern of effects emerged with the use of AP-7. Three-way repeated measures ANOVA showed no significant difference between treatments (sucrose × diazepam) \(F_{1,28} = 0.88; \ P > 0.05\), levels of anxiety (LA × HA) \(F_{1,28} = 2.40; \ P > 0.05\) or a significant interaction between treatment × level of anxiety \(F_{1,28} = 0.39; \ P > 0.05\). Main effects were obtained for condition (withdrawal × AP-7 effects) \(F_{1,28} = 58.19; \ P < 0.0001\) and a significant interaction was also observed between condition × treatment \(F_{1,28} = 7.65; \ P < 0.005\). No significant interactions were obtained for condition × anxiety level \(F_{1,28} = 0.06; \ P > 0.05\) or among condition × treatment × anxiety level \(F_{1,28} = 0.31; \ P > 0.05\). Again, post hoc tests indicated that AP-7 decreased the amplitude of the startle response of sucrose and diazepam withdrawn groups, regardless of the level of anxiety presented.

Experiment V: Effects of the blockade of cIC glutamate receptors on the amplitude of AEPs induced by auditory stimuli in diazepam pre-treated rats

This experiment investigated whether the decrease in startle amplitude after cIC microinjections of glutamate antagonists was due to a decrease in aversive states induced by withdrawal or due simply to the ability of the drugs to interfere with the processing of the startle-auditory stimuli in this structure. For this purpose, 40 naïve Wistar rats were submitted to chronic treatment with sucrose or diazepam and tested after 48 h of withdrawal. Immediately before the test, the animals were anesthetized with an i.m. injection of 0.2 ml of xylazine (chlorhydrate, 20 mg/ml), a drug that does not interfere with the acoustically evoked activity in the cIC (Nwabueze-Ogbo et al., 2002). Surgery, drug microinjections, and histology were performed exactly as in experiment IV except that, in this case, an electrode glued to a guide cannula, surgically implanted in the cIC, was used for both drug stimulation and AEP registration.

Experimental apparatus. Evoked potentials were recorded as the voltage difference between the tips of the bipolar electrode implanted in the cIC. The signal registered was fed into an amplifier (Lynx, São Paulo, Brazil, TX001, bandwidth set to 10–100 Hz) through two noiseless shielded cables (passed through a hole in the roof of a Faraday cage). The output of each amplifier was connected to one of the four channels on an analogue/digital converter (CAD 1236) plugged into a PC. The data acquisition sweep began 10 ms before the onset of the sound stimulation (latency to switch on the sound plus sound propagation) and continued until 200 ms after it. Signal analyzer software (Bio-spector, Lynx, São Paulo, Brazil) was programmed to sum 100 individual evoked potentials in order to improve the signal/noise ratio. This set of data was monitored on the computer screen. The computer output was graphically displayed on an XY plotter (Hewlett-Packard 1100, Palo Alto, CA, USA). Data on AEP were stored on the hard disk of the computer and transferred to Excel (Excel, Microsoft Corp., Mountain View, CA, USA) tables for off-line visualization and analysis. In order to maintain body temperature at 37/38 °C the animals were placed on a heat-pad and maintained in this condition during the experiments. A 7.5 W red bulb on the top of the testing box was switched on during the experimental sessions. A loudspeaker, located 10 cm behind the wire mesh cage, delivered continuous background noise (55 dB sound pressure level [SPL]). Acoustic stimuli (clicks) were delivered via two piezoelectric speakers (12 fl, 200 W, LeSon, São Paulo, Brazil) mounted on the lateral walls of the sound insulating box, 15 cm away from the heat-pad. The AEP stimulus was a pure tone with an intensity of 92.5 dB, 3000 Hz. The AEP was recorded after each of the 100 auditory stimuli and the mean value was obtained at the end of the sessions. Software and an appropriate interface (Lynx, São Paulo, Brazil) controlled the presentation and sequencing of the acoustic stimuli. SPLs were measured at the level of the ears of the animals using a Bruel and Kjær 0.125-in. microphone and a type 2636 measuring amplifier (DK-2580; Naerum, Denmark). Calibration procedures were conducted before the experiments to ensure equivalent sensitivities be-
fore each session. The experiments were monitored live through a video camera mounted on the rear wall, inside the testing box, located approximately 12 cm above the floor.

**Experimental procedures.** On the day of the test, the animals were anesthetized with xylazine, 15 min prior to the physiological saline microinjections. Following these procedures, each rat was placed in the testing cage, connected to the recording system, and the baseline AEP was taken. Fifteen minutes after AP-7 or GDEE was administered, and after another 10 or 5 min delay, respectively, the animals were retested. Four groups were formed in a within-subjects design: suc/H11003 physiological saline followed by GDEE 15 min later (n=11); suc/H11003 physiological saline followed by AP-7 15 min later (n=11); Dzp/H11003 physiological saline followed by GDEE 15 min later (n=8); and Dzp/H11003 physiological saline followed by AP-7 15 min later (n=8).

**Statistical analysis.** Data are presented as mean±SEM. Raw data from AEP were averaged for each animal across the entire session and normalized through square root transformation. After that, a three-way repeated measures ANOVA was performed with the oral (sucrose×diazepam) and central (GDEE×AP-7) treatments as the between factors and the condition (baseline×test) as the within-repeated variable. When appropriate (P<0.05) all statistical analyses were followed by the Fisher LSD post hoc test.
Results.

Three-way repeated measures ANOVA showed no significant effects (P > 0.05) for the antagonists on the AEP recorded in the cIC of anesthetized sucrose or diazepam-withdrawn rats (treatments: F 1,34 = 0.57, antagonist effects: F 1,34 = 0.70, treatments × antagonist effects: F 1,34 = 0.05, conditions × treatments: F 1,34 = 0.30, conditions × groups × antagonist effects: F 1,34 = 0.02), as seen in Fig. 6. Data were analyzed by three-way repeated measures ANOVA. The tests were followed by Fisher LSD post hoc test.

DISCUSSION

It is well known that both humans and laboratory animals, when facing fear- and anxiety-inducing stimuli or after administration of anxiogenic drugs, evoke an increased startle response (Davis et al., 1979, 1993; Liebsch et al., 1998b; Winslow et al., 2002). Similarly, the startle response is overdone in clinical patients with anxiety disorders (Grillon et al., 1998). In the present study, LA and HA rats submitted to an acute oral intake of sucrose or diazepam evoked different startle responses. HA sucrose pre-treated animals displayed reduced startle amplitude when compared with LA rats. Acute oral diazepam intake was unable to influence startle response to an acoustic stimulus in HA rats in the same way that it did in the LA group. We suggest that the increased levels of fear elicited by EPM aversive cues, in a biased version of EPM in which access to the closed arms was prevented, could account for the absence of the effects of diazepam on the startle response, in the same way that the drug maintains its anxiolytic effects on inhibitory avoidance tasks, but not on the one-way flight response, when tested in the elevated T-maze. In other words, HA rats may exhibit a sense of fear close to the panic state, in which diazepam is ineffective, in contrast to LA rats that may evoke an affective state associated more with generalized anxiety (Graeff et al., 1998). On the other hand, the sedative effects of acute diazepam could account for the decreased startle amplitude observed in LA rats. The absence of similar results in LA rats submitted to chronic diazepam treatment and tested under the effects of the drug (as seen in experiment II) seems to corroborate this hypothesis. The question that remains is why the sedative effect of diazepam was not observed in HA rats tested under acute diazepam effects.

Concerning the chronic effects of and withdrawal from diazepam, analysis of the startle response amplitude in LA sucrose and diazepam pre-treated rats showed that there was no statistically significant difference between the two groups tested under the effect of the drug. Thus, diazepam was not able to promote its anxiolytic effects in chronically pre-treated rats that do not normally display anxiety-like behavior, a result quite similar to that noted for rats tested under the acute effects of diazepam. On the other hand, HA diazepam-withdrawn rats (those that presented inborn elevated anxiety levels) exhibited reduced startle amplitude when tested under the effect of diazepam. This reduction in amplitude in HA rats is specifically related to the anxiolytic effects of the drug, since the sedative effects were absent after 18 days of chronic treatment. As a matter of fact, it has been demonstrated that in both humans and laboratory animals the efficacy of an anxiolytic...
may depend upon the basal level of anxiety (Green, 1991; Lader, 1991; Griebel, 1995; Rodgers et al., 1997). Otherwise, HA rats withdrawn from diazepam showed an increase in startle response when compared with the same animals tested under the effect of diazepam. However, this increase in startle response did not differ from that achieved in LA rats tested under the effects of chronic diazepam. With regard to this point, our data show that, despite that diazepam does not affect the startle response in LA rats, withdrawal from chronic treatment promotes increased levels of aversion in rats selected for their inborn anxiety.

Based on the results discussed above, it is possible that the increases in startle amplitude observed in HA rats tested under diazepam withdrawal may be due to the effect of reductions in the anxiety-like effects observed in the chronic condition. However, the results obtained for USV showed that the affective state elicited in the former is quite different from that observed in the latter. In fact, HA diazepam withdrawn-rats emitted twofold more 22-kHz aversive frequencies, formerly known as alarm calls (for a review see Litvin et al., 2007), than the LA withdrawn-rats. These results indicate that, although no difference was found between HA and LA withdrawn-rats in terms of startle response, the emotional states of the two groups are qualitatively different.

The influence of midbrain structures on anxiety induced by drug withdrawal has been suggested (Cabral et al., 2006; Fontanesi et al., 2007; Souza-Pinto et al., 2007). In fact, in a previous study in our laboratory it was shown that withdrawal from the Bzp diazepam enhances Fos-protein immunolabeling in almost all brainstem areas examined. Among the investigated areas, the cIC showed the most prominent Fos neural activation (Fontanesi et al., 2007).

The IC is a midbrain structure that, in conjunction with the dPAG, deep layers of superior colliculus, dorsomedial hypothalamus and amygdala, composes the well-known brain aversion system. This part of the CNS forms a set of brain structures primarily involved in the modulation/expression of unconditioned anxiety and fear behaviors (Graeff, 1990; Brandão et al., 1999, 2003). It is well known that the cIC is primarily involved in the processing of auditory stimuli and also in the integration of acoustic information of an aversive nature (Brandão et al., 1993, 1999). For instance, in a recent study in our laboratory, we showed that freezing behavior induced by 22-kHz ultrasound signals can be suppressed by cIC microinjections of the Bzp midazolam or the GABA-A receptor agonist muscimol (Nobre and Brandão, 2004), indicating that the expression of unconditioned freezing in response to ultrasound signals is modulated by GABA–Bzp mechanisms.

So, based on the assumptions described above, it is possible that the same neurobiological substrates that are activated in animals facing anxiety-provoking stimuli could be overactive during Bzp withdrawal, as suggested in a recent study (Fontanesi et al., 2007). In this case, our results indicate the possibility that the anxiety-like behavior promoted by withdrawal from diazepam could be the result of alterations in primitive midbrain systems that regulate the expression of unconditioned fear and anxiety behaviors in the early stages. The fact that similar emotional negative states are attenuated by anxiolytic drugs seems to support the view that common neurobiological substrates may be affected by both experiences, anxiety-like states elicited by fear stimuli and drug withdrawal.

It has been postulated that the effects of withdrawal following chronic Bzp are the result of a homeostatic imbalance promoted by chronic drug treatment. In fact, as part of a compensatory mechanism for diazepam-induced chronic enhancement of GABAergic inhibition, excitatory mechanisms (e.g. those mediated by glutamate) become more sensitive (Stephens, 1995). In line with this view, the study of Souza-Pinto et al. (2007) showed that the negative-reinforcing effects of diazepam withdrawal induce place-preference courses with enhanced glutamatergic neurotransmission in brainstem structures that normally modulate unconditioned fear and anxiety-like behaviors, since the aversive withdrawal effects are reversed after antagonism of the dPAG glutamate receptors. If so, it is likely that, during Bzp withdrawal, decreased GABA inhibition associated with enhanced glutamate excitatory activity in brainstem structures that commonly modulate fear and anxiety-like behaviors, such as the dPAG and IC, could underlie the increased levels of unconditioned fear and anxiety observed in patients withdrawn from Bzp substances, as well as in laboratory animals.

The results obtained in the present study seem to strengthen this hypothesis. Indeed, the increased levels of anxiety observed in HA diazepam-pretreated rats, as revealed by increased startle amplitude, reverted to the control condition following AMPA and NMDA receptor antagonism. The different potency of effects on the startle response obtained by the two drugs was likely due to the distinctive dynamics of the two receptors in the cIC, as the AMPA receptors (fast-acting) mediate rapid-onset, depolarizing postsynaptic currents and NMDA receptors (long-lasting) mediate depolarizing currents with more gradual onset and longer duration (Sanchez et al., 2007). In this context, one point deserves attention: the drugs were also effective in reducing the startle response in control animals. In fact, an anxiolytic-like effect of glutamate antagonist locally administered in several brain regions, such as some hypothalamic nuclei, dPAG and IC, has already been described in previous studies (Cardoso et al., 1994; Pandóssio and Brandão, 1999; Molchanov and Guimarães, 2002; Jardim et al., 2005). This is a well known effect of this class of drugs. For instance, the study of Kapus et al. (2008) showed that the use of AMPA receptor antagonists may inhibit anxiety-like behavior in rodents, independently from their motor depressant activity. In the same way, it has been suggested that the activation of NMDA receptors causes increases in anxiety-like behavior elicited by cat odor in rats later tested in the EPM (Adamec et al., 1998). The blockade of these receptors with MK-801, AP7, or CPP, prevents this enhancement, reflecting the anxiolytic action of the drugs. In the field of drug abuse, the first experimental evidence suggesting the importance of the
glutamate receptors in the production of the symptoms of Bzp withdrawal was the demonstration that the intracerebral injection of AMPA or NMDA receptor antagonists reduces the appearance of these signs in mice (Stephnum and Tursky, 1993). In previous studies in our laboratory (Souza-Pinto et al., 2007) we showed that inhibition of glutamatergic neurotransmission in the dPAG reduces the consequences of diazepam withdrawal in rats. In harmony with all these findings the present results show that infusions of glutamate antagonists reduce the startle amplitude regardless of the anxiety level in both control and withdrawn rats. However, it is important to note that antagonism of the long-lasting NMDA receptors AP-7 was two-fold more potent at reducing the startle response in diazepam-withdrawn rats than in control specimens, showing that glutamatergic excitatory neurotransmission in cIC is more prominent in the former than in the latter. The fast-acting characteristics of AMPA receptors could account for the inability of GDEE to promote similar effects.

One problem still remains: it is possible that the pharmacological manipulations used in this study simply interfered with the processing of the auditory stimulus applied, decreasing the normal processing of auditory information in the cIC. In order to respond this problem, another experiment was performed and the results showed that, curiously, neither GDEE nor AP-7 was able to change the amplitude of AEP elicited by an auditory stimulus in slightly anesthetized rats tested under diazepam withdrawal. The real nature of this phenomenon could not be explained and additional studies must be conducted in order to understand this discrepancy. However, it seems that withdrawal acquired its negative affective qualities through association with the characteristics of the stimuli and the context in which the tests were performed. In other words, the effects of glutamate antagonists on the emotionality of rats under diazepam withdrawal can be assessed only in awake animals.

CONCLUSION

In summary, the current results raise some important points regarding Bzp dependence and withdrawal. As suggested by other studies the present findings showed that the anxiolytic effect of a Bzp is a function of the animal’s basal anxiety levels, such that the absence of this state renders the drug impotent. In the same way, rebound anxiety seems to be absent in rats selected for low inborn anxiety levels (LA) and chronically treated with diazepam, showing that dependence was not achieved in these animals. Finally, since Bzp withdrawal may function as an unconditioned stressor, as with other drugs of abuse, the negative affective states observed on withdrawal from diazepam could be a by-product of GABA–glutamate imbalance in brain systems that mediate unconditioned fear and anxiety behaviors, since the blockade of AMPA and NMDA glutamate receptors in the cIC clearly reduced the aversion promoted by diazepam withdrawal.

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