

Analysis of the chronic intake of and withdrawal from diazepam on emotional reactivity and sensory information processing in rats

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Abstract

It has been demonstrated that, on abrupt withdrawal, patients with chronic exposure can experience a number of symptoms indicative of a dependent state. In clinical patients, the earliest to arise and most persistent signal of withdrawal from chronic benzodiazepine (Bzp) treatment is anxiety. In laboratory animals, anxiety-like effects following abrupt interruption of chronic Bzp treatment can also be reproduced. In fact, signs that oscillate from irritability to extreme fear behaviours and seizures have been described already. As anxiety remains one of the most important symptoms of Bzp withdrawal, in this study we evaluated the anxiety levels of rats withdrawn from diazepam. Also studied were the effects on the motor performance and preattentive sensory gating process of rats under diazepam chronic treatment and upon 48-h withdrawal on three animal models of anxiety, the elevated plus-maze (EPM), ultrasonic vocalizations (USV) and startle + prepulse inhibition tests. Data obtained showed an anxiolytic- and anxiogenic-like profile of the chronic intake of and withdrawal from diazepam regimen in the EPM test, 22-KHz USV and startle reflex. Diazepam chronic effects or its withdrawal were ineffective in promoting any alteration in the prepulse inhibition (PPI). However, an increase of PPI was achieved in both sucrose and diazepam pretreated rats on 48-h withdrawal, suggesting a procedural rather than a specific effect of withdrawal on sensory gating processes. It is also possible that the prepulse can function as a conditioned stimulus to informing the delivery of an aversive event, as the auditory startling-eliciting stimulus. All these findings are indicative of a sensitization of the neural substrates of aversion in diazepam withdrawn animals without concomitant changes on the processing of sensory information.

Keywords: Anxiety, Diazepam withdrawal, Elevated-plus-maze, Prepulse inhibition, Ultrasonic vocalization.

Abbreviations: Bzp, benzodiazepines; EPM, elevated plus-maze test; PPI, prepulse inhibition; USV, ultrasonic vocalizations.

1. Introduction

It is well recognized that, in addition to its peculiar somatic signs, withdrawal from most drugs of abuse including alcohol, psychostimulants and nicotine, opiates and benzodiazepines (Bzp) can promote common disturbances in affective states and emotionality (Markou et al., 1998). In the case of Bzp, chronic treatment was supposed not to cause dependence. However, it has been demonstrated that, on abrupt withdrawal, patients with chronic exposure can experience a number of symptoms indicative of a dependent state. The earliest to arise and most persistent signal of withdrawal from chronic Bzp treatment is anxiety (Marks, 1978), although clinical reports have also denounced the appearance of high levels of fear in Bzp withdrawn patients (Rosebush and Mazurek, 1996).

In laboratory animals, similar aspects of the withdrawal phenomena can be reproduced. In fact, signs that oscillate from irritability to extreme fear behaviours and seizures have already been described (Ashton, 2005; Emmett-Oglesby et al., 1983a; Lacerra et al., 1999; Owen and Tyrer, 1983; Petursson and Lader, 1981a, 1981b; Woods et al., 1987). For example, rats withdrawn from diazepam exhibited high levels of aversion when submitted to the elevated plus-maze (EPM) (File and Andrews, 1991; Martijena et al., 1996), increased levels of ultrasonic vocalizations (USV) on 22-KHz frequencies and enhanced startle response amplitude to an auditory startling-eliciting stimulus (Vivian et al., 1994; Vivian and Miczek, 1993). A similar level of defensive behaviour is observed in animals faced with several dangerous situations such as exposure to predators (Blanchard et al., 1991), foot-shocks (De Vry et al., 1993) or playback of 22-KHz aversive frequencies (Brudzynski and Chiu, 1995). However, in most of the studies cited above chronic intraperitoneal injection, a procedure known to

enhance anxiety levels of rats (Griebel et al., 1994), was used as the choice of drug delivery.

The amplitude of startle reflex response is attenuated when the startling-eliciting stimulus, as an auditory one, is preceded by a weak prepulse non-startling stimulus. This phenomenon is termed prepulse inhibition (PPI) and has been observed across a wide range of species (Geyer and Ellenbroek, 2003; Hoffman and Ison, 1980). However, the influence of the anxiolytic effects of chronic treatment with diazepam or the aversive effects of withdrawal from this Bzp on the processing of a prepulse signal has not yet been investigated.

Based on these assumptions, the present study was designed to provide a broad perspective on the potential influence of chronic effects of and withdrawal from oral intake of diazepam on emotional reactivity as well as on the processing of sensory information in rats. The first two parts of this study were designed to assess the effectiveness of chronic oral treatment with diazepam and its withdrawal in promoting anxiolytic and aversive effects, respectively, in animals tested on the EPM test and 22-KHz USV elicited by foot-shock. In the third part, acoustic startle responses and PPI were assessed with the same approach, as these measures have been used to index motor performance and sensory-motor gating, respectively.

2. Materials and methods

2.1. Animals

One hundred and sixteen male Wistar rats, weighing 100–110 g at the beginning of the treatment, from the animal house of the campus of Ribeirão Preto, University of São Paulo, were used. They were housed in groups of four in Plexiglas-walled cages, lined with wood shavings that were changed every three days, maintained in a 12:12

dark/light cycle (lights on 07:00 h) at $24 \pm 1^\circ\text{C}$. The rats were given free access to food and partial access to water. Before the beginning of the treatments, the animals had a three-day habituation period to the lodging conditions, deprivation and drinking procedures. The experiments reported in this article were performed in compliance with the recommendations of SBNeC (Brazilian Society for Neuroscience and Behavior), which are in accordance with the rules of the U.S. National Institutes of Health Guide for Care and Use of Laboratory Animals.

2.2 Administration of diazepam by oral route

The oral intake procedure as the choice for drug administration was chosen mainly to avoid the anxiogenic-like effects promoted by chronic systemic drug injections (Griebel et al., 1994). Moreover, in a previous study by our laboratory (data not published yet), we showed that rats receiving 18 days of chronic i.p. saline injections displayed high anxiety levels when compared with animals that received chronic oral sucrose or those that remained undisturbed in the vivarium and were tested on plus-maze. This demonstrated that the oral drug administration procedure is supposed to produce more precise data than those approaches using i.p. injections.

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 hours of water deprivation (19:00 to 9:00), followed by 10 hours of water ad libitum (9:00 to 19:00). This procedure began three days before the start of the 18-day treatment, as an acclimitization period for the animals to the drinking procedure. Diazepam was separately dissolved in a concentration of 10 mg/ml of saline plus propilenoglycol (5%) and offered, once daily, in a dose of 1 ml/kg diluted in a solution of 2 ml of tap water added to sucrose (5%) plus propilenoglycol (5%), which was also used as control

solution. The solutions were made available to the animals in glass pipettes of 5 ml, at the end of the daily period of water deprivation (09:00 h), and were delivered during each of the 18 days of treatment. Except for the first two days of treatment, in which control and experimental solutions were available for 30 minutes, the animals that did not drink the sucrose or diazepam solutions for 10 minutes were removed from the experiments. This was necessary to avoid prolonging the deprivation period. Experimental sessions were conducted for 30 minutes (termed the chronic effects condition, in which the animals were tested during the effect of the drug) or 48 hours after the last oral drug intake (the withdrawal condition, in which the animals were tested free of the drug).

2.3. Experiment I

Withdrawal-induced anxiety in rats tested in the EPM

2.3.1 Experimental apparatus

This experiment was designed to observe the influence of chronic intake of diazepam or its interruption on the anxiety levels of rats tested in the EPM. The EPM was made of wood 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). To avoid falls, a 3-mm wooden rim surrounded the open arms. The EPM was located inside a refrigerated room with a constant noise (50 dB). Behaviour in the EPM was documented by a camera (Everfocus, Duarte, CA, USA) linked to a monitor and video-cassette, outside the experimental room, which allowed the recordings to be analysed later. Luminosity at the level of the open arms of the maze was 20 lux. Four groups of animals were arranged in order to avoid the same animal being exposed twice to the maze (n = 12 for each group). The

groups were named sucrose chronic effects, diazepam chronic effects, sucrose withdrawal and diazepam withdrawal.

2.3.2 Experimental procedure

The rat was placed at the centre of the EPM facing an enclosed arm and allowed to explore the environment for 5 minutes. The apparatus was cleaned with 20% ethanol and water before the next rat was placed in the EPM. The measures taken were the traditional indexes of exploration expressed as percentage of entries (with the four paws) into and time spent on the open arms in relation to total number of entries and time, respectively. The total number of entries into the closed arms was also recorded. Animals of the sucrose or diazepam groups were tested for 30 minutes or 48 hours after the last sucrose or diazepam oral intake, respectively. Each animal was tested only once.

2.3.3 Statistical analysis

The data obtained in the EPM were analysed using one-way ANOVA with the treatments (sucrose or diazepam) as the independent factor, and the number of closed arm entries, percentage of entries and time spent in the open arms as the dependent factor. Newman-Keuls post-hoc comparisons were carried out whenever significant overall F-values were obtained. In all cases a probability level of $p < 0.05$ was considered to be significant.

2.4. Experiment II

Effects of diazepam withdrawal on USV induced by foot-shocks

2.4.1 Experimental apparatus

The apparatus used for recording and analysing the USV has been described in detail in previous reports (Bassi et al., 2007; Nobre et al., 2003; Nunes Mamede Rosa et al., 2005). Briefly, it consisted of a testing box ($25 \times 15 \times 12$ cm) made of steel bars spaced approximately 12 mm apart. This experimental chamber was situated inside a larger, padded, echo-free (sound-attenuated) and ventilated chest ($60 \times 40 \times 45$ cm) with a 28 W red light bulb located at the top of the chamber. For recording and analysis of USV, four ultrasound bat detectors (Ultra Sound Advice, London, UK) were mounted in the lateral and rear walls of the chamber and linked to a multichannel audio apparatus with 20-KHz low band pass (cut-off) filter (Noldus Instruments, Amsterdam, The Netherlands) and then fed into a computer. During each testing session each bat detector was tuned to a different frequency (20, 22, 24 or 26 KHz) to record the USV.

The choice of the frequencies above was based on previous studies which showed that rats exposed to dangerous situations display an enhancement of the emissions of 22- and 24-kHz USV that do not differentiate from each other (Nobre and Brandão, 2003; Nunes Mamede Rosa et al., 2005). The same studies showed that the 20- and 26-kHz frequencies are emitted less. The sessions consisted of placing the animals individually inside the experimental chamber for 5 minutes of acclimitization. After this, they received just one foot-shock (0.6 mA, 1 s), produced by a shock generator (Insight, Sao Paulo, Brazil). A video-camera linked to a TV monitored the behaviour for the overall 10 minutes period of recording after the foot-shock was delivered. The number of calls and duration of USV emission in seconds were analysed by a sonograph (Ultravox signal analysis work station, Noldus Instruments, Amsterdam, The Netherlands), stored on a hard disc and subsequently transferred to tables in a spreadsheet programme (Excel; Microsoft Corp., Mountain View, CA, USA) for off-

line analysis. Each frequency input in each of the four microphones used was calibrated before the session began with the use of an audio generator (GW, 1MHz, NJ, USA).

2.4.2 Experimental procedure

Two independent groups of animals were used to evaluate the effects of diazepam withdrawal on foot-shock-induced ultrasonic vocalizations. As in experiment I, the animals were submitted to an 18-day chronic intake of sucrose or diazepam solutions ($n = 14$ in each group). Twenty minutes after the last sucrose or diazepam intake the animals were handled for 5 minutes and then placed in the experimental box, where they remained undisturbed for an additional 5 minute period of acclimitization. Immediately after the foot-shock was delivered ultrasonic measures were registered for 10 minutes (chronic sessions). Forty-eight hours later, using the same protocols, the same animals were submitted to the withdrawal sessions.

2.4.3 Statistical analysis

Data are reported as mean + SEM. Separate two-way repeated measures (RM) ANOVA for comparisons between values recorded 30 minutes or 48 hours after withdrawal were performed, with the treatments as the independent variables and the frequency as the dependent repeated variable. In addition, comparisons were carried out to evaluate the differences between the exposure to chronic and withdrawal effects in the same group (sucrose or diazepam). Post-hoc comparisons were carried out whenever significant overall F-values were obtained. A value of $p < 0.05$ was considered significant.

2.5. Experiment III

Effects of diazepam chronic intake and withdrawal on startle response and PPI

2.5.1 Experimental apparatus

The rats were placed in a stabilimeter, which consisted of a wire-mesh cage ($16.5 \times 5.1 \times 7.6$ cm) suspended within a PVC frame ($25 \times 9 \times 9$ cm), which was attached to the response platform with four thumbnail-screws. The stabilimeter and platform were located inside a ventilated plywood sound-attenuating chamber ($64 \times 60 \times 40$ cm). The floor of the stabilimeter consisted of six stainless steel bars 3.0 mm in diameter and spaced 1.5 cm apart.

The startle reaction of the rats generated a pressure on the response platform and analogue signals were amplified, digitized and analysed by the software of the startle measurement system (Insight, Sao Paulo, Brazil), which also controlled all parameters of the session (intensity of the acoustic stimulus, inter-stimulus interval, etc.). The startle amplitude was recorded within a time window of 200 ms after the onset of the startle stimulus. Two loudspeakers located 10 cm above the floor, on each lateral side of the acoustic isolation chamber, were used to deliver the prepulse stimulus, the acoustic startle stimuli and a continuous background noise (white noise, 50 dB).

Calibration procedures were conducted before the experiments to ensure equivalent sensitivities of the response platforms over the test period. Animal behaviour was recorded by a video camera (Safety View, São Paulo, Brazil) located behind the stabilimeter, allowing the discrimination of all possible behaviours, with the signal being relayed to a monitor in another room via a closed circuit. A red light bulb (15 W) was located on top of the experimental box, 50 cm above the stabilimeter, to provide illumination for the camera.

2.5.2. Experimental procedure

Startle response and PPI was tested in a simple paradigm routinely used in our laboratory (Cabral et al., 2006; Nunes Mamede Rosa et al., 2005). The acoustic startle test session for measuring the PPI consisted of four parts. The first was a 5 minutes period of acclimitization to the startle test chamber. No acoustic startle stimuli were presented during this period. The second part consisted of 20 presentations of a pulse alone (70 dB of pure tone, 3,000 Hz frequency, 20 ms duration). The third part consisted of 20 presentations of an acoustic startle stimulus (pulse, 110-dB, 50-ms bursts of white noise having a rise-decay time of 5 ms). The fourth part tested the prepulse modulation of the acoustic startle (prepulse + pulse). It consisted of 20 trials of an acoustic startle stimuli preceded 30 ms by a pure tone (prepulse) with a 30 seconds inter-trials interval.

In this study, a pure tone was used as a prepulse stimulus since we have noted that the use of a white noise, but not a pure tone, activates both the affective and sensory channels, as this type of sound seems to promote aversion. The purpose of the procedures above was to determine the effects of the acclimitization to the continuous presentation of the prepulse, startle or prepulse + startle stimuli on the amplitude of startle responses. The inter-stimulus interval of 30 s used between trials was based on a previous study by our laboratory (Cabral et al., 2006; Nunes Mamede Rosa et al., 2005).

The testing session lasted 35 minutes. The startle response amplitudes obtained from this experiment were stored on a hard disc and subsequently transferred to tables in a spreadsheet programme for off-line analysis. Two groups were formed: sucrose (n = 21) and diazepam (n = 19). The same animals were tested 30 minutes (during chronic drug effects) and 48 h after the last diazepam oral intake (during withdrawal effects).

2.5.3 Statistical analysis

The results from startle and PPI tests are all reported as means \pm SEM. Startle responses were averaged for each animal across the entire session and used as the data for statistical analysis. A two-way RM ANOVA was conducted on the startle and PPI, separately, with the groups (sucrose x diazepam) as the independent and the amplitude of startle responses obtained during chronic or withdrawal conditions as the dependent repeated factors. The results of the PPI are reported as % PPI = $100 - (100 \times \text{mean startle magnitude on 'pre-pulse + pulse' stimulus divided by the mean startle magnitude on 'pulse' alone})$. All statistical analyses were followed, when appropriate, by Newman-Keuls test. $P < 0.05$ was considered to be significant.

3. Results

3.1 Withdrawal-induced anxiety in rats tested in the EPM

One-way ANOVA showed significant differences on the percentage of entries ($F_{3,44} = 13.66$, $p < 0.0001$) and time spent in the open arms ($F_{3,44} = 16.52$, $p < 0.0001$), and on the number of closed arms entries ($F_{3,44} = 3.65$, $p < 0.05$). Post-hoc analysis revealed that rats tested 30 minutes after the last chronic oral intake of diazepam showed little anxiety-like behaviour (Figure 1) when compared with the control group, as revealed by the increase in the mean percentage of time spent in the open arms displayed by these animals ($F_{3,44} = 9.86$, $p < 0.0001$). On the other hand, 48 h of diazepam withdrawal enhanced the levels of anxiety-like behaviour as revealed by the reduction in both the percentage of entries and time spent in the open arms of the EPM. One-way ANOVA analysis also demonstrated a significant effect of the treatments on the number of closed arms entries ($F_{3,44} = 3.65$, $p < 0.05$). Post-hoc test revealed that this

effect was due to the ability of diazepam to increase the motor behaviour of rats tested under the effects of this drug (chronic sessions).

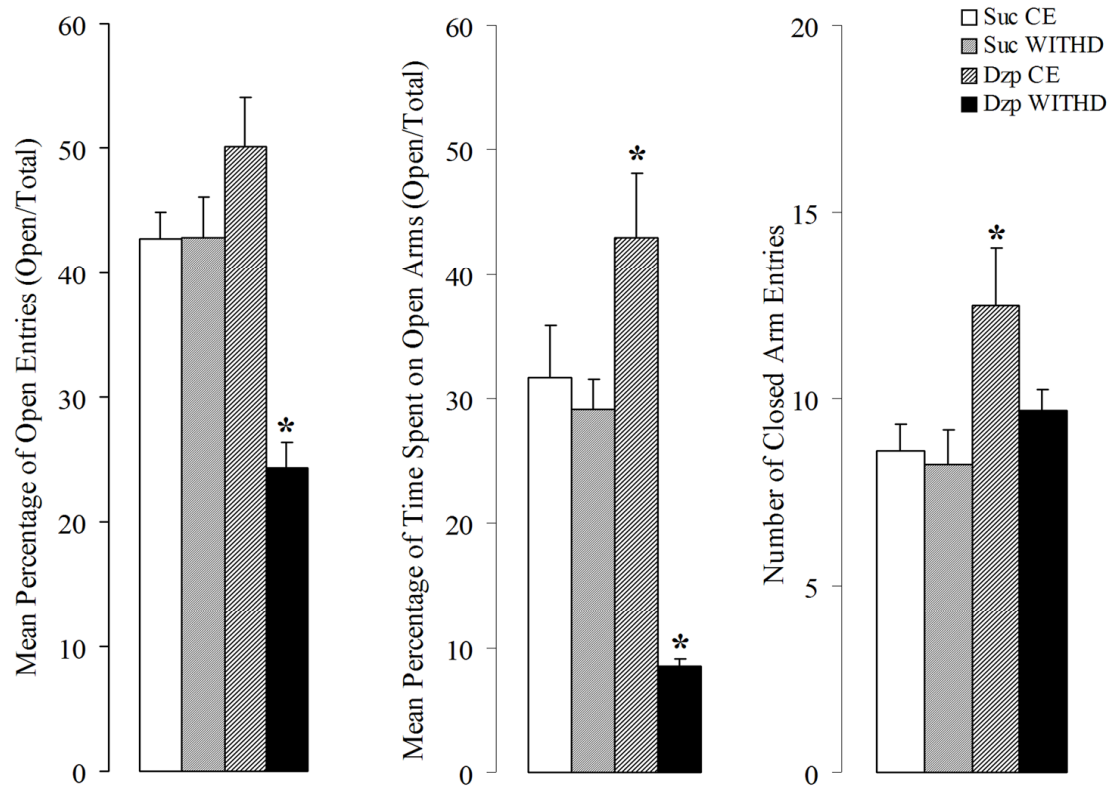


Fig 1

Figure 1: Mean (+SEM) percentage of entries and time spent in the open arms, and mean number of closed arms entries of rats chronic treated with (Suc) or diazepam (Dzp) and tested 30 min (chronic effects - CHRON) after the last oral intake or on 48 h withdrawal (WITHD). * Significant difference between Suc or Dzp groups when compared with their respectively CHRON or WITHD conditions. One-way ANOVA followed by Newman-Keuls post-hoc.

3.2. Effects of diazepam withdrawal on USV induced by foot-shocks

Figures 2 and 3 show the mean number and duration of USV emitted at the frequencies of 20, 22, 24 and 26 KHz soon after foot-shocks were delivered 30 minutes (chronic condition) or 48 h (withdrawal condition) after the interruption of the

treatments with sucrose or diazepam oral intake. The number and duration of USV at 20 to 26 KHz was subjected, independently, to a two-way RM ANOVA (treatment \times frequency or condition \times frequency) with the frequencies as the repeated measures.

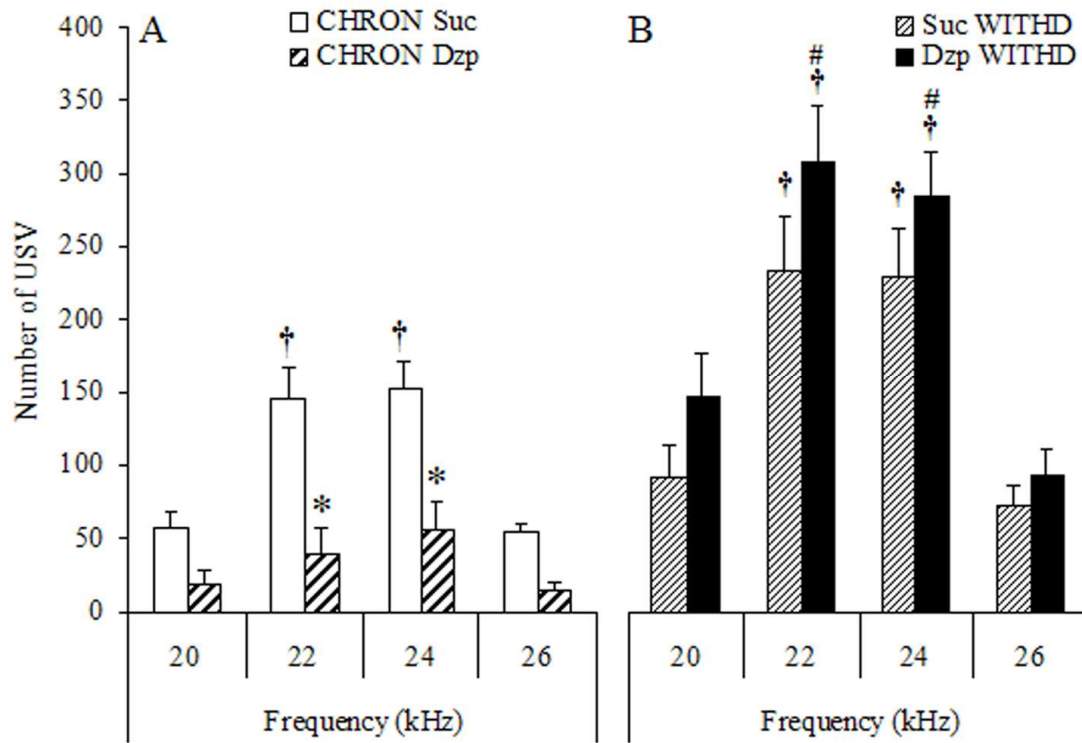


Figure 2: Mean (+SEM) of the number of USV emitted at the frequencies of 20–26 KHz during the first 10 min after foot-shock delivery in rats tested 30 min after the last drug intake (2A, CHRON) or 48 h withdrawal (2B, WITHD) from sucrose (Suc) or diazepam (Dzp). *Significant difference between Dzp and Suc pretreated animals in the same frequency on the same condition CHRON or WITHD. †Significant difference between frequencies on the same group (Suc or Dzp) and condition (CHRON or WITHD) when compared with the 20 KHz frequency. #Significant difference between WITHD or CE conditions on the same frequency on the same group (Dzp or Suc). Two-way RM ANOVA followed by the Newman-Keuls test.

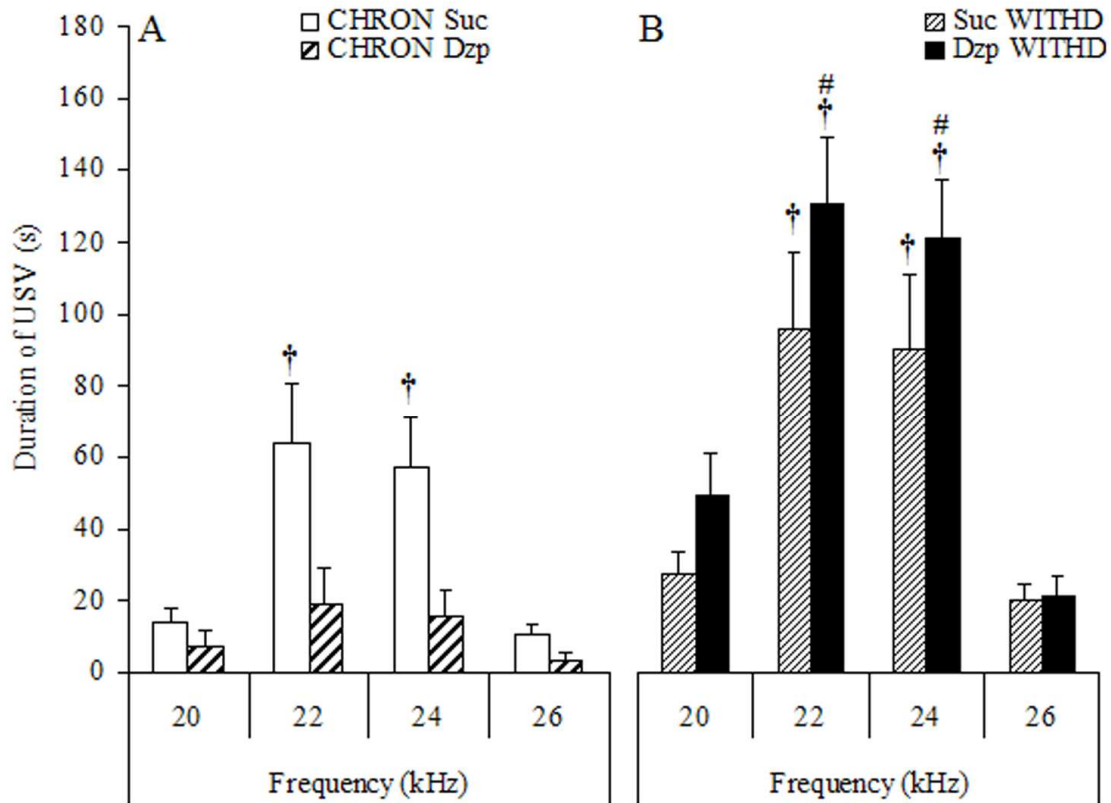


Figure 3: Mean (+SEM) duration of vocalizations emitted at the frequencies of 20–26 KHz during the first 10 minutes after foot-shock delivery of rats tested at 30 min (3A, CHRON) or 48 h withdrawal (3B, WITHD) from sucrose (Suc) or diazepam (Dzp). †Significant difference between frequencies on the same group (Suc or Dzp) and condition (CHRON or WITHD) when compared with the 20 KHz frequency. #Significant difference between WITHD or CHRON conditions on the same frequency on the same group (Dzp or Suc). Two-way ANOVA followed by the Newman-Keuls test.

This analysis showed significant differences between treatments on the number (treatments, $F_{1,26} = 15.83$, $p < 0.0005$; conditions, $F_{3,78} = 19.15$, $p < 0.0001$; treatments \times conditions, $F_{3,78} = 4.82$, $p < 0.005$) and duration (treatments, $F_{1,26} = 4.95$, $p < 0.05$; conditions, $F_{3,78} = 10.40$, $p < 0.0001$; treatments \times conditions, $F_{3,78} = 3.70$, $p < 0.05$) of vocalizations of rats tested 30 minutes after the last chronic intake of sucrose or diazepam solutions. On the other hand, two-way RM ANOVA showed no significant effects of the treatments or interactions between the treatments and frequencies during

withdrawal. However significant effects were obtained on the frequencies (number of USV, $F_{3,78} = 44.52$, $p < 0.0001$; duration of USV, $F_{3,78} = 32.58$, $p < 0.0001$).

Additional comparisons also showed significant differences on the emission of USV of animals belonging to the sucrose group during withdrawal, when compared with the measures obtained in the same rats during the chronic condition (number, $F_{3,78} = 36.65$, $p < 0.005$; duration, $F_{1,26} = 18.47$, $p < 0.0001$). A similar pattern of effects was obtained in animals 48 h from diazepam withdrawal, when compared with the indices collected on the same rats under chronic diazepam effects. In fact, two-way RM ANOVA revealed significant differences in the number (treatments, $F_{1,26} = 45.30$, $p < 0.0001$; conditions, $F_{3,78} = 26.98$, $p < 0.0001$; treatments \times conditions, $F_{3,78} = 13.47$, $p < 0.0001$) and duration (treatments, $F_{1,26} = 34.09$, $p < 0.0001$; conditions, $F_{3,78} = 27.15$, $p < 0.0001$; treatments \times conditions, $F_{3,78} = 15.49$, $p < 0.0001$) of USV.

Post-hoc testing showed that animals under the effects of diazepam decreased their ability to emit 22-KHz USV in the presence of aversive foot-shocks, when compared with the sucrose controls, 30 minutes after the last oral consumption of the drug. The results obtained after 48 h showed no differences between diazepam and sucrose groups on withdrawal. However, diazepam pretreated rats presented an enhancement of the emission of the aversive 22-KHz USV during withdrawal when compared with the measures registered in the same rats tested under diazepam chronic effects.

3.3 Effects of diazepam chronic intake and withdrawal on startle response and PPI

The magnitude of the startle reflex and the prepulse modulation of startle in the two groups of rats (sucrose and diazepam) are illustrated in Figure 4. Two-way RM

ANOVA revealed that there was no significant difference in groups ($F_{1,38} = 1.85$, $p > 0.05$). However, statistically significant effects were obtained for conditions (chronic \times withdrawal; $F_{1,38} = 6.87$, $p < 0.05$).

The treatments \times condition interaction was also significant ($F_{1,38} = 5.53$, $p < 0.05$). Post-hoc tests revealed that diazepam treatment does not change the amplitude of startle if the animals are tested 30 minutes (chronic effects) after the last oral administration (4A) but significantly enhances this response if the rats are tested at 48 h after diazepam withdrawal (panel 4B). Two-way RM ANOVA showed no differences in the prepulse on startle response between the groups tested ($F_{1,38} = 1.85$, $p > 0.05$) or even significant interaction between treatment \times conditions ($F_{1,38} = 0.015$, $p > 0.05$; panels A and B). However a significant difference was achieved on condition ($F_{1,38} = 16.41$, $p < 0.05$).

Newman-Keuls post-hoc showed that, independent of the treatment employed, animals of both sucrose and diazepam groups present enhanced PPI during withdrawal, when compared with the measures obtained 30 minutes after the last control or drug solutions intake (4C).

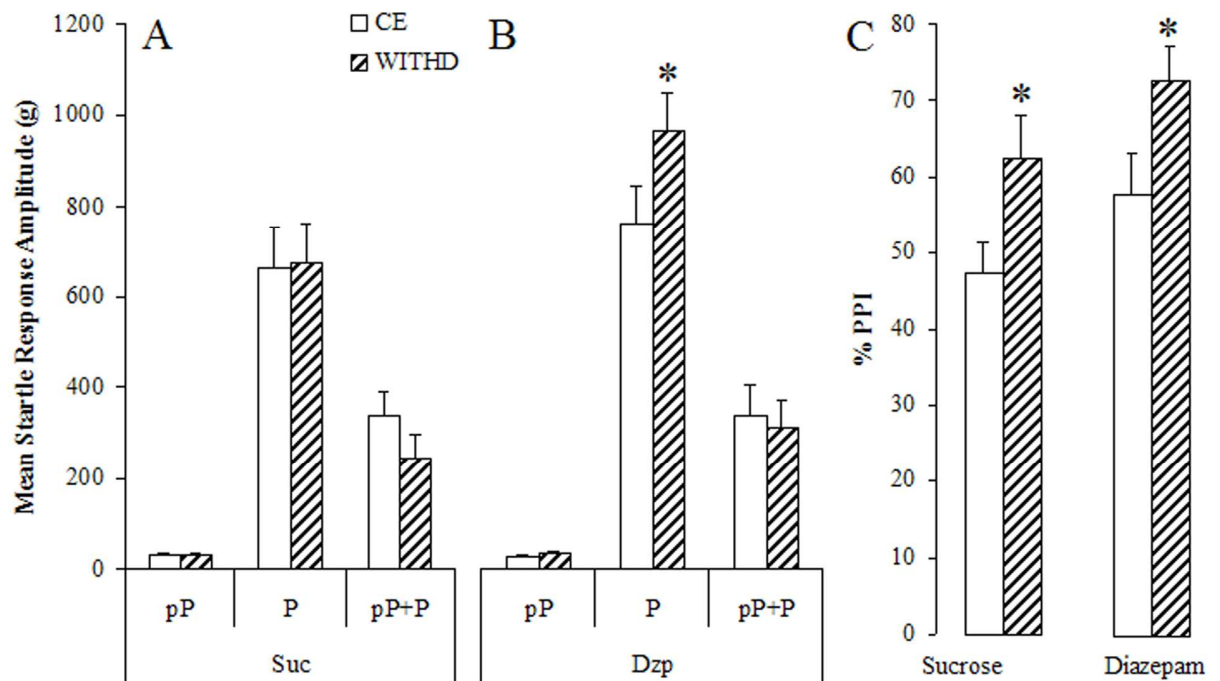


Figure 4: Mean (+SEM) of startle response amplitude of rats chronically treated with sucrose (4A) or diazepam (4B) and tested under chronic effects (CHRON) or during withdrawal (WITHD). Histograms show the amplitude of startle response induced by a prepulse (pP), pulse (P) or prepulse + pulse presentation (pP+P). The percentage of PPI is showed right (4C). *Significant difference on startle response amplitude during withdrawal when compared with that observed in rats under chronic sucrose or diazepam effects (CHRON). Data on pP, P and pP+P were analysed by a two-way RM ANOVA. For the percentage of PPI one-way RM ANOVA was used. The tests were followed by Newman-Keuls post-hoc.

4. Discussion

The interruption of a long period of treatment with Bzp produces a great probability of developing withdrawal symptoms both in humans and in laboratory animals (Ladewig, 1984; Lukas and Griffiths, 1982). In fact, high anxiety levels have been reported as a common symptom in patients withdrawn from Bzp and avoidance of these unpleasant symptoms seems to negatively reinforce continuous Bzp use, one of the main components of craving for the drug (Busto et al., 1986; Busto and Sellers, 1991). An increase in anxiety-like states is also observed in rats undergoing

spontaneous diazepam withdrawal and submitted to several types of animal models of anxiety (Emmett-Oglesby et al., 1983a, 1983b; File, 1990; Greenblatt and Shader, 1974).

As anxiety remains one of the main symptoms present during diazepam withdrawal syndrome, the EPM has frequently been employed to evaluate the degree of drug withdrawal-induced anxiety (Begg et al., 2005; Izzo et al., 2001; Martijena et al., 1996). The EPM was first validated as a test of anxiety-related behaviour for rats (Pellow and File, 1986) and it is frequently considered as a 'primer' test for measuring this type of behaviour. In our study, the interruption of the chronic oral regimen of diazepam caused an anxiogenic effect in the EPM test in the same fashion as those previously reported after abrupt interruption of alcohol chronic treatment (Cabral et al., 2006). In fact, withdrawn animals tend to spend more time inside and also to enter less times into the open arms of the EPM, when compared with controls. Thus, diazepam withdrawal induced anxiety-like effects that remain at least 48 h after the interruption of the treatment. On the other hand, anxiolytic-like effects associated with an increase in the percentage of time spent in the open arms of the maze was observed in rats under diazepam effects, tested 30 minutes after the last oral intake.

The same pattern of results was obtained in the USV test. There was a reduction of emission of 22-KHz USV elicited by foot-shocks in rats under diazepam effects and an increase in this measure on 48 h withdrawal. Our results lend further support for the hypothesis that rats exposed to unavoidable stressful stimuli display a series of behavioural and physiological responses, often including intense 22-KHz USV that serves as cries of distress (Cuomo et al., 1988). That this type of response reflects an aversive emotional state relies on the fact that drugs that commonly reduce anxiety also promote anxiolytic-like effects in this model (Cuomo et al., 1988; Vivian and Miczek,

1993). Moreover, it is well established that such vocalizations serve as an alarm signal warning conspecifics of the presence of imminent danger (Blanchard et al., 1991) as rats submitted to pure 22 KHz tone (Nobre and Brandão, 2004) or playback of 22 KHz (Brudzynski and Chiu, 1995) display characteristic defensive behaviour.

The effects of diazepam withdrawal on ultrasound emissions in rodents were previously demonstrated in some studies that used acoustic stimuli and foot-shocks as ultrasound-elicited stimuli, mainly using the subcutaneous or intraperitoneal injections as the method of drug delivery (Kikusui et al., 2001; Miczek and Vivian, 1993; Nielsen and Sanchez, 1995; Vivian et al., 1994). As it was observed that the chronic intraperitoneal injection of a placebo solution elicits, by itself, anxiety in rats tested in the EPM (Griebel et al., 1994), in this study we used the voluntary oral intake as the method of choice. In a previous study we have demonstrated that this method elicits low anxiety levels in rats treated chronically (Fontanesi et al., 2007). Our results showed higher levels of aversion in withdrawn rats when compared with controls, revealed by their increased levels of vocalizations. Interestingly, there was an increase in the 22-KHz USV in control animals treated with sucrose solutions when they were tested 48 h after the first session. This effect is probably due to the contextual fear conditioning these animals experienced as they returned to the same experimental box where they previously received foot-shock.

The acoustic startle response is a reflexive neurobehavioural response mediated at the brain stem level (Koch, 1999; Lee et al., 1996) and is known to be affected by the withdrawal from chronic diazepam treatment (Chugh et al., 1992; Miczek and Vivian, 1993; Rasmussen et al., 1993). In our study rats withdrawn from oral diazepam intake increased their magnitude of startle response, in a similar manner to that verified during withdrawal from other drugs of abuse, such as alcohol (Chester et al., 2004; Pohorecky

et al., 1976), morphine (Harris and Gewirtz, 2004; Kalinichev and Holtzman, 2003) and nicotine (Acri, 1994; Helton et al., 1993).

The results on PPI showed that rats undergoing diazepam treatment or withdrawn from this drug present a similar percentage of inhibition of startle response, when compared with the sucrose controls. The same pattern of effects was observed in other studies that showed no influence of drug withdrawal on the processing of sensory information (Abduljawad et al., 1997; Cabral et al., 2006; Fendt and Mucha, 2001; Peleg-Raibstein et al., 2006). However, there was an unexpected enhancement of the PPI during withdrawal in both sucrose and diazepam groups. As no differences were observed between treatments it is possible that the PPI enhancement during withdrawal in both control and diazepam groups reflects the effects of repetition of the test.

Prepulse inhibition has been hypothesized to be automatic, or ‘hard-wired’, because it is unlearned (Graham et al., 1975), requires only midbrain and lower brain structures (Leitner and Cohen, 1985), occurs in decorticate animals (Davis and Gendelman, 1977) and also occurs in human adults while they are asleep (Silverstein et al., 1980). However, some studies have initiated some controversy on the automatic nature of the prepulse inhibition. In fact, evidence from studies demonstrated that, in humans, the short-lead interval PPI effect can also be modulated by selective attention, suggesting that it is not an entirely automatic process in all situations (DelPezzo and Hoffman, 1980; Hackley et al., 1987; Hackley and Graham, 1987). Moreover, other studies hypothesized that the accuracy of discriminating the prepulse stimulus is highly correlated to the degree of suppression of the startle reflex (Filion and Cirrani, 1994; Mussat-Whitlow and Blumenthal, 1997; Norris and Blumenthal, 1995).

In a recent study, Zou et al. (2007) showed that the magnitude of the startle reflex response could be strongly inhibited when a prepulse stimulus, that becomes a

conditioned signal for informing aversive events, is presented immediately before the startling-eliciting stimulus. In addition, the possibility that the prepulse inhibition phenomenon can be modulated by selective attention in some situations relies on the fact that the impairment of some prosencephalic structures, such as the prefrontal cortex or amygdala, mainly involved in the modulation of the cognitive and emotional aspects of behaviour, significantly reduce PPI in rodents (Bubser and Koch, 1994; Koch and Bubser, 1994; Li and Shao, 2003; Swerdlow et al., 1995, 2001). In our study, the increased inhibition of the startle reflex observed on the withdrawal condition in both sucrose- or diazepam-withdrawn animals, seems to be an effect of the aversive learning induced on animals that were submitted repeatedly to the same experimental condition. In this situation, the prepulse could be conditioned to informing the auditory-startling aversive stimulus, promoting an enhancement on the effects of the prepulse, in the same way as a conditioned aversive stimulus, such as foot-shocks can do (Zou et al., 2007). The trends towards difference between diazepam pretreated and sucrose animals observed during withdrawal could be solely due to the high level of anxiety present in withdrawn rats that favour the aversive conditioning in these animals.

In summary, the present results raise some important points in the field of the neurobiology of benzodiazepine withdrawal. It is possible that the same neurobiological substrates that are activated to promote anxiety-like behaviours induced by external aversive stimuli could be overactive during benzodiazepine withdrawal. In a recent study (Fontanesi et al., 2007) we showed high levels of neural activation in brainstem areas mainly involved in the modulation/expression of defensive behaviours in rats withdrawn from diazepam. Chemical or electrical stimulation of these same regions elicits a pattern of performance (Brandão et al., 2003, 2005) similar to that which we obtained in the present study: decreased entries and time spent in the open arms of the

plus-maze; enhanced emission of ultrasonic vocalizations on 22-KHz aversive frequency; and increased amplitude of startle response. In this case, our results indicate the possibility that the anxiety-like behaviour promoted by withdrawal from diazepam could be the result of alterations in primitive systems that regulates the expression of fear behaviours early, with little or no influence of the prosencephalic structures that modulate cognitive processes.

Finally, the fact that similar emotional negative states are attenuated by anxiolytic drugs seems to support the view that similar neurobiological substrates may be affected by both experiences, anxiety-like states elicited by fear stimuli and drug withdrawal. The fact that prepulse inhibition was not changed in withdrawal animals reinforces the idea that brain structures involved in the processing of sensory and cognitive information have little or no influence in this state.

5. Conclusions

Our results confirm previous reports showing that the abrupt interruption of the chronic intake of diazepam elicits high levels of aversion in rats, as revealed in all anxiety tests used in this study. A similar pattern of defensive behaviours is also observed in animals promptly faced with dangerous situations, such as exposure to a predator or cues signalling an aversive event, showing that diazepam withdrawal and fear-elicited stimuli could activate the same neurobiological substrates without harming the processing of sensory and cognitive information.

Acknowledgements

Research supported by grants from FAPESP (Proc. n° 04/02859-0).

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