Brainstem areas activated by diazepam withdrawal as measured by Fos-protein immunoreactivity in rats

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ABSTRACT

In the 1970s, chronic treatment with benzodiazepines was supposed not to cause dependence. However, by the end of the decade several reports showed that the interruption of a prolonged treatment with diazepam leads to a withdrawal syndrome characterized, among other symptoms, by an exaggerated level of anxiety. In laboratory animals, signs that oscillate from irritability to extreme fear-like behaviors and convulsions have also been reported. In recent years many studies have attempted to disclose the neural substrates responsible for the benzodiazepines withdrawal. However, they have focused on telencephalic structures such as the prefrontal cortex, nucleus accumbens and amygdala. In this study, we examined the Fos immunoreactivity in brain structures known to be implicated in the neural substrates of aversion in rats under spontaneous diazepam-withdrawal. We found that the same group of structures that originally modulate the defensive responses evoked by fear stimuli, including the dorso-medial hypothalamus, the superior and inferior colliculus and the dorsal periaqueductal gray, were most labeled following diazepam withdrawal. It is suggested that an enhanced neural activation of neural substrates of fear in the midbrain tectum may underlie the aversive state elicited in diazepam-withdrawn rats.

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dPAG

1. Introduction

Benzodiazepines compounds are widely used for the treatment of anxiety and sleep disorders and are also prescribed for their sedative, muscle relaxant and anticonvulsant properties (Woods et al., 1987; Bateson, 2002). Initially, chronic treatment with benzodiazepines was supposed not to cause dependence. However, it has been demonstrated that, on abrupt withdrawal from benzodiazepine, chronic exposure patients can experience a number of symptoms indicative of a dependent state. Among the symptoms raised, an exaggerated level of anxiety (rebound anxiety), more intense than that presented during the prescription of the drug, has been mentioned (Chouinard, 2004; Rynn and Brawman-Mintzer, 2004). Additionally, clinical reports have denounced the appearance of high levels of fear in patients experiencing benzodiazepine withdrawal (Rosebush and Mazurek, 1996). In the laboratory, similar aspects of the withdrawal phenomena can be reproduced in animals. Actually,
signs that oscillate from irritability to extreme fear behaviors and convulsions have already been described (Petursson and Lader, 1981a,b; Owen and Tyrer, 1983; Emmett-Oglesby et al., 1983; Lacerra et al., 1999; Woods et al., 1987; Ashton, 2005).

The great majority of methods used in the investigation of the neurobiological mechanisms implicated in the production of fear and anxiety as, for example, chemical or electrical stimulation or lesions of brain structures, can provide only indirect evidences on the cerebral operation. In this case, the use of immunohistochemical techniques, such as the Fos protein detection, has been employed because it allows a clear visualization of the neurons activated during the presentation of a specific fear stimulus (Dragunow and Faull, 1989; Reiman et al., 1989; Bullitt, 1990). In fact, Fos-like immunoreactivity has been used as a marker of the neuronal activation that can be precipitated by a wide range of stimulants, such as, for example, brain electrical stimulation (Sandner et al., 1992; Vianna et al., 2003), local injection of neurotransmitters (Stone et al., 1991; Richard et al., 1992; Ferreira-Netto et al., 2005), stress induced by immobilization (Imaki et al., 1992; Honkanienmi et al., 1992), pain (Bullitt, 1990) and abstinence of several types of drugs of abuse such as opiates (Hayward et al., 1990), psychostimulants (Persico et al., 1995), alcohol (Moy et al., 2000) and benzodiazepines (Dunworth et al., 2000). Using this technique, it has been shown that some prosencephalic structures are activated by benzodiazepine withdrawal, such as the shell region of the nucleus accumbens (Dunworth et al., 2000), one of the most important areas involved with the expression of motivational factors linked to drugs of abuse. This type of activation is also produced by anxiogenic stimuli that induce increase in dopamine release and induction of Fos immunoreactivity in this structure (Abercrombie et al., 1989; Smith et al., 1997). In the same context, a simple exposure to the elevated plus-maze produces great Fos labeling in limbic areas (Silveira et al., 1993; Sandner et al., 1993) related with the production of the behavioral and autonomic responses associated with the expression of fear.

Drug withdrawal may function as an unconditioned stressor, and as such could activate a particular set of structures involved with the organization of fear states, particularly those belonging to the well-known brain aversion system, such as the amygdala, dorsal periaqueductal gray (dPAG) (the main effector pathway of the defensive behavior responses), superior and inferior colliculi (structures included in the processing of visual and auditory information of aversive nature, respectively) and the medial hypothalamus (mainly tied to the production of the somatic responses of fear) (Gray, 1982; Graeff, 1990; Brandão et al., 1999, 2005). To examine this possibility, in this study we analyzed whether the expression of fear behaviors observed in rats abruptly withdrawn from diazepam could lead to a consequent activation of the same brain structures that modulate the expression of the defensive behavior of animals exposed to dangerous situations such as, for example, exposure to the open arms of the elevated plus-maze, among others. To this end, we used the immunohistochemical technique for the detection of Fos-protein expression in selected structures of diazepam-withdrawn rats.

The great majority of studies in the literature that investigate the chronic effects of a drug of abuse have mainly used intraperitoneal injections as the method of delivery of the drug. However, it was demonstrated that rats under chronic systemic injections of placebo solutions tend to present an increase in the anxiety levels when tested in the elevated-plus-maze (Griebel et al., 1994). In this case, it could be argued that the aversion promoted by the exposure to chronic systemic injections could lead to an increase in the number of Fos-like immunoreactive neurons in structures that normally modulate this class of emotional responses. To circumvent this problem, in this work, 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.

Fig. 1 – Percentage of entries in the open arms (A), number of enclosed arms entries (B) and percentage of time spent in the open arms (C) of the elevated plus-maze of rats that did not receive any treatment (No Treat) and rats treated chronically p.o. with sucrose or diazepam. As ANOVA showed no significant differences between the two control groups used in this study, all the differences shown in the graphs are relative to comparisons between the diazepam-treated rats and those belonging to the sucrose group. Independent groups of diazepam treated animals were tested at the dependence (30 min after the last drug intake—Dzp DEP) or withdrawal (48 h after the last drug intake—Dzp W). Data are presented as mean±S.E.M. One-way ANOVA followed by post-hoc Newman–Keuls; p<0.05.
2. Results

2.1. Plus-maze

One-way ANOVA revealed that the chronic treatment with sucrose, per se, did not promote any effect on the behavior of rats after the interruption of the long-term treatment, as there were no observed significant differences in any of the registered measures on the behavior of rats of this group, when compared with those that did not suffer any intervention (No Treat). On the other hand, significant group differences in both the percentage of entries [F (3,72)=5.35; p<0.005] and on the percentage of time spent in the open arms [F (3,72)=18.55; p<0.001] were observed in animals under diazepam effects (Dzp DEP) or during withdrawal (Dzp W), when compared with those that received sucrose only. Newman–Keuls post-hoc showed that the chronic oral intake procedure was effective in producing a significant anxiolytic-like effect in rats tested 30 min after the last oral administration of diazepam (Dzp DEP), as revealed by the increase in the percentage of entries (Fig. 1A) and in the total ‘open arms’ time (Fig. 1C). An anxiogenic-like effect was achieved in rats on 48-h withdrawal from diazepam (Dzp W), as these animals stayed less time in the open arms of the maze (Fig. 1C) than the animals of the sucrose group. The number of ‘closed arms’ entries [F (3,72)=3.22; p>0.05] showed a trend towards significance (p=0.055) to this condition among the groups tested. As shown in Fig. 1B, this effect was due to a tendency of rats under diazepam effect (Dzp DEP) to increase motor activity, when compared with the sucrose control group.

2.2. Fos immunoreactivity

Statistical analysis of the effects of the diazepam withdrawal on Fos-protein expression was conducted in brain areas of 22 animals (n=11 for group) randomly chosen from each of the sucrose- and diazepam-withdrawal groups (Dzp W) (see Table 1). The mean number of Fos-protein immunoreactivity in neuronal nuclei is shown in Figs. 2, 4 and 6, for each brain region studied.

<table>
<thead>
<tr>
<th>Structures</th>
<th>Sucrose</th>
<th>Diazepam</th>
<th>p&lt;0.05</th>
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<td>7.20±1.27</td>
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<td>3.40±0.45</td>
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Table 1 – Mean (±S.E.M.) number of Fos-positive neurons/0.1 mm² in brain regions of 48-h diazepam-withdrawn rats perfused 2 h after plus-maze exposure.

Number of Fos-positive neurons per region on the left and right sides were counted using a computerized image analysis system (Image Pro Plus 4.0, Media Cybernetics). Asterisks indicate the regions in which withdrawn-rats displayed differential Fos-expression (p<0.05) in relation to control group (sucrose). Differences between groups were analyzed with the Student t-test for each region in study. PrL, prelimbic cortex; CG1 and CG2, cingulate areas 1 and 2, respectively; CeA, central nucleus of the amygdala; MeA, medial nucleus of the amygdala; BLA, basolateral nucleus of the amygdala; CA1, CA2 and CA3, hippocampal areas 1, 2 and 3, respectively; AcbC, nucleus accumbens (core region); AcbSh, nucleus accumbens (shell region); PV, paraventricular thalamic nucleus; PaV, paraventricular hypothalamic nucleus; AH, anterior hypothalamus; LH, lateral hypothalamus; DMPAG, dorsomedial hypothalamic gray; DLPAG, dorsolateral periaqueductal gray; LPAG, lateral periaqueductal gray; VLPAG, ventrolateral periaqueductal gray; DR, dorsal raphe nucleus; MnR, median raphe nucleus; SC, superior colliculus; IC, inferior colliculus; LC, locus ceruleus; Cun, cuneiform nucleus.
2.2.1. Telencephalon
As revealed in Figs. 2 and 3, withdrawal from diazepam did not cause important Fos expression in almost all telencephalic regions. In fact, the Student t-test revealed significant differences in the Fos expression only in the pre-limbic cortex (PrL) \([t_{20}=3.73; p<0.05]\) (Fig. 3, upper right). Otherwise, many other areas mainly involved in withdrawal of drugs of abuse, as the core (AcbC) and shell (AcbSh) regions of the nucleus accumbens, showed no alterations on Fos-protein immunoreactivity (Fig. 3, bottom right).

2.2.2. Diencephalon
High neural activation was observed in diencephalic structures (Figs. 4 and 5). Forty-eight hours of diazepam withdrawal was effective in promoting a robust increase in Fos expression in the anterior (AH) \([t_{20}=2.86; p<0.05]\) and lateral hypothalamus (LH) (Fig. 5, upper right) \([t_{20}=2.75]\) and also in the dorsomedial (DMH) \([t_{20}=5.03; p<0.05]\) nuclei of the hypothalamus of the abstinent rats (Fig. 5, bottom right). Student t-test revealed that the comparisons between the withdrawal and sucrose groups reached marginal significance \((p=0.056)\) of the withdrawal on the expression of Fos positive neuronal nuclei in the paraventricular region of the hypothalamus (PaV).

2.2.3. Mesencephalon
Statistical analysis indicated significant differences in Fos expression in almost all mesencephalic areas studied (Figs. 6 and 7), as the dorsomedial [DMPAG: \(t_{20}=7.74; p<0.05]\], dorsolateral [DLPAG: \(t_{20}=6.11; p<0.05]\], lateral [LPAG: \(t_{20}=5.35; p<0.05]\] and ventrolateral [VLPAG: \(t_{20}=3.87; p<0.05]\] columns of the periaqueductal gray and dorsal raphe nucleus [DR: \(t_{20}=3.24; p<0.05]\] (see Fig. 7, upper right panel). Student t-test also showed significant differences in the superior [SC: \(t_{20}=7.51; p<0.05]\] and inferior [IC: \(t_{20}=8.28; p<0.05]\] colliculi (Fig. 7, bottom right) and cuneiform nucleus [Cun: \(t_{20}=5.07; p<0.05]\]. Interestingly, of all mesencephalic structures studied, only locus ceruleus showed a significant decrease in Fos-protein expression, when compared with the control group [LC: \(t_{20}=3.44; p<0.05]\].

3. Discussion
The interruption of a long period of treatment with benzodiazepines produces great probability of developing withdrawal symptoms in humans or in laboratory animals (Lukas and Griffiths, 1982; Ladewig, 1984). In fact, anxiety-like states have been reported as a common symptom in benzodiazepine-withdrawn patients. Avoidance of these unpleasant symptoms seems to negatively-reinforce continuous benzodiazepine use and is 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 under spontaneous diazepam-withdrawal and submitted to several types of animal models of anxiety (Greenblatt and Shader, 1974; Emmett-Oglesby et al., 1983; File, 1990). In our study, the interruption of the chronic regimen of diazepam caused a significant decrease in the percentage of time spent in the open arms of the plus-maze. This anxiogenic effect promoted by diazepam withdrawal was accompanied by significant Fos-positive immunoreactivity in telencephalic, diencephalic and mesencephalic areas including all columns of the periaqueductal gray, the deep layers of the superior colliculus, the central nucleus of the inferior colliculus,
the medial aspects of the dorsal raphe nuclei, the anterior, lateral and doro-medial nuclei of the hypothalamus and the prelimbic cortex, all of them mainly linked to the modulation of the emotional, autonomic and motor expression of the fear-motivated behaviors. For example, the prelimbic cortex, amygdala and hypothalamus, in conjunction with other structures, are part of many well-defined anxiety- and fear-related circuits in the brain, so that anxiogenic drugs that promote high levels of aversion also significantly activate these circuits (Charney et al., 1998; Gorman et al., 2000; Gray and McNaughton, 2000; Rosen and Schulkin, 1998). Parts of the prefrontal cortex, including the prelimbic region, have been reported to be involved in the production of fear- and anxiety-related behaviors (Espesjo, 1997; Jinks and McGregor, 1997; LeDoux, 1995). Additionally, many studies on acute fear have proposed that brain areas such as the amygdala, hippocampus, hypothalamus and periaqueductal gray are putatively involved in fear processing (Fendt and Fanselow, 1999; Graeff, 1994; Gray and McNaughton, 2000; LeDoux, 1995). Others have implicated the periaqueductal gray, amygdala, medial hypothalamus, raphe nuclei, superior and inferior colliculus and locus ceruleus in the expression of the fear behaviors (Graeff, 1990, 1994; Brandão et al., 1999, 2003, 2005).

The activation of the amygdala results in behavioral and physiological responses associated with anxiety and panic-like behavior. It is intriguing that no changes in Fos-expression in this region were observed in our study. However, a similar result was also obtained by Borlikova et al. (2006), who showed that a single alcohol-withdrawal experience does not induce significant Fos expression in this area, contrasting with a significant Fos expression following repeated withdrawal procedures. The activation of the paraventricular hypothalamic nucleus (PaV) promotes release of a variety of hormones that are involved in the neuroendocrine and autonomic responses to fear and psychological stress, whereas the activation of the lateral hypothalamicus seems to be important for the cardiovascular expressions of fear and anxiety. Also, in laboratory animals, electrical stimulation of the anterior hypothalamic nucleus elicits hissing and threat postures characteristic of defensive behaviors. Electrical or chemical stimulation of some mesencephalic structures belonging to the well-known brain aversion system, such as the superior and inferior colliculus and periaqueductal gray, also promote defensive behaviors in rats (Brandão et al., 1999, 2005). The activation of brainstem structures was also observed in several studies using Fos-protein expression as a neuronal marker of anxiety-like states (Senba et al., 1993; Sandner et al., 1993; Silveira et al., 1995; Beck and Fibiger, 1995; Beckett et al., 1997; Canteras and Goto, 1999), and after intraperitoneal injection of

**Fig. 3** – Photomicrograph of Fos-like immunoreactive cells (dark dots) in coronal sections showing Fos expression through the prelimbic cortex (PrL, above) and on the core (AcbC) and shell (AcbSh) regions of the nucleus accumbens (below) of rats submitted to spontaneous diazepam-withdrawal (Dzp W, right) and tested 48 h after the interruption of the treatment, when compared with its controls (Suc, left). Cg1 = cingulate cortex, area 1; fmi = forceps minor of the corpus callosum; Cl = claustrum; IL = infralimbic cortex; LV = lateral ventricle; LacbSh = lateral accumbens shell; aca = anterior commissure, anterior part; ec = external capsule.
**Fig. 4** – Number of Fos-immunoreactive neurons in the diencephalic structures of 48-h diazepam-withdrawn rats submitted to the plus-maze. Data are expressed as mean ± S.E.M. of Fos-positive cells in 0.1 mm² area of tissue. The number of Fos-positive neurons per region was bilaterally counted using a computerized image analysis system (Image Pro-Plus 4.0, Media Cybernetics). * Significant difference on the number of Fos-positive cells in each structure studied between diazepam (Dzp W) and sucrose groups. The analysis was performed with the use of the Student t-test. The level of significance was set at p<0.05.

drugs that elicit panic-like symptoms (Singewald and Sharp, 2000). All these common sets of structures involved in the expression of the emotional, autonomic and motor components of fear-related behaviors were also strongly activated in rats suffering withdrawal which were submitted to the plus-maze, showing that the same neuronal changes verified during the presentation of fear stimuli seems to be occurring during diazepam withdrawal.

The main neurobiological mechanism associated with the effects of drugs of abuse has been the dopaminergic mesolimbic system and its connections with the basal prosencephalon (Koob, 1992; Koob and Le Moal, 1997). In this context, in a previous experiment, Dunworth et al. (2000) investigated whether some diazepam-withdrawal symptoms could be lessened by prior withdrawal experience in mice undergoing a single or repeated flumazenil-precipitated diazepam withdrawal. In the third experiment of this study, they evaluated the Fos-protein expression in brain areas that they speculated would be important in either seizure sensitivity or the expression of an aversive state. The immunohistochemistry results showed a significant increase in Fos expression in the shell region of the nucleus accumbens of mice submitted to a single withdrawal experience; contrasting with our findings that showed no significant alteration on this measure in the same area. On the other hand, no differences were observed after flumazenil injection in rats undergoing repeated diazepam-withdrawal experience. Concerning this discrepancy, we must pay attention to some methodological aspects that could clarify the differences obtained between the two studies. Most importantly, as revealed above, on Dunworth’s experiments, Fos immunohistochemistry assays were performed in mice through the flumazenil-precipitated diazepam-withdrawal procedure, in contrast with the spontaneous diazepam-withdrawal method used in our experiments. This difference is seminal to understand the increase of Fos expression observed in the former study. Concerning this point, the study of Motzo et al. (1997) demonstrated that a challenge administration of diazepam inhibits dopamine output in the nucleus accumbens. On the other hand, the discontinuation of long-term treatment with diazepam does not alter the basal dopamine release in this region. However, a single intraperitoneal injection of flumazenil (in a dose that has no effect on the basal dopamine levels) is effective in enhancing the release of this neurotransmitter in the nucleus accumbens of rats chronically treated with diazepam and tested after either 6 h or 5 days of withdrawal. In this case, we could argue that the enhanced Fos expression observed in the Dunworth’s study could be due to a hyperfunctioning of the dopaminergic neurons on the accumbens, following flumazenil injection. Besides, the flumazenil concentration (20 mg/kg) used in the experiment of Dunworth et al. was much more larger than that noted in the study of Motzo (4 mg/kg). In this case, an increase on the basal activity of the dopaminergic cells that could contribute to promote Fos expression on the accumbens, could not be disregarded.

The absence of Fos expression in the nucleus accumbens in the present study may be related to the notion that benzodiazepine withdrawal recruits neural substrates different from the dopaminergic one (Lingford-Hughes and Nutt, 2003) to promote its effects. Indeed, changes in the GABA system have been postulated to underlie diazepam-withdrawal syndrome (Miller et al., 1988; Galpern et al., 1991; Toki et al., 1996). Secondary alterations in other neurotransmitter systems as, for example, the glutamatergic and serotonergic mechanisms have also been considered (Allison and Pratt, 2003; Andrews et al., 1997). Additionally, we have found that the inhibition of the glutamatergic neurotransmission through local injections of AMPA-kainate or NMDA antagonists in the dPAG reduce the fear behaviors in diazepam-withdrawn rats, implicating the excitatory amino acids of the dPAG in the modulation of the aversive states induced by diazepam withdrawal (Souza-Pinto et al., 2007). As the mechanisms that modulate fear are primarily located in the brainstem, it is possible that these changes take place initially in the mesencephalon. This is supported by the pronounced Fos-positive immunoreactivity in structures basically involved in the modulation of fear behaviors, such as the periaqueductal gray and the superior and inferior colliculi of rats under diazepam withdrawal. Thus, it is likely that the fear elicited by diazepam withdrawal is the result of the activation of the neural substrates of aversion in the brainstem, such as the defense reaction elicited by stimulation of the dPAG (Brandão et al., 1999, 2005). On the other hand, prosencephalic structures, such as amygdala, are involved in the modulation of the anxiety promoted by associative learning, such as that generated in aversive conditioning. In support of the present results, we have found that rats under ethanol withdrawal had an increase in
Fig. 5 – Photomicrograph of Fos-like immunoreactive cells (dark dots) in coronal sections showing Fos expression through the paraventricular (PvA), anterior (AH) and lateral (LH) regions of the hypothalamus (above), and the dorsomedial hypothalamic nucleus (below) of rats submitted to spontaneous diazepam-withdrawal (Dzp W, right) and tested 48 h after the interruption of the treatment, when compared with its control (Suc, left). f = fornix; 3V = third ventricle; VMH = ventromedial hypothalamus; opt = optic tract; mt = mamillothalamic tract; ic = internal capsule.

Fig. 6 – Number of Fos-immunoreactive neurons in the mesencephalic structures of 48-h diazepam-withdrawn rats submitted to the plus-maze. Data are expressed as mean ± S.E.M. of Fos-positive cells in 0.1 mm² area of tissue. The number of Fos-positive neurons per region was bilaterally counted using a computerized image analysis system (Image Pro-Plus 4.0, Media Cybernetics). * Significant difference on the number of Fos-positive cells in each structure studied between diazepam (Dzp W) and sucrose groups. The analysis was performed with the use of the Student t-test. The level of significance was set at p<0.05.
reactivity to the electrical stimulation of the dPAG along with a reduction in their ultrasonic vocalizations (a phenomenon usually observed during exposure to an intense uncontrollable stressor), showing that ethanol withdrawal sensitizes the substrates of fear at the level of dPAG (Cabral et al., 2006).

In summary, neural substrates of fear seem to be recruited by the withdrawal from diazepam intake in rats, as revealed by the Fos-protein immunohistochemistry assay. In this context, this is the first report showing that an increase of the activation of the neural substrates of fear in the midbrain tectum may underlie the aversive states elicited by diazepam withdrawal.

4. Experimental procedures

4.1. Animals

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 changed every 3 days, maintained in a 12:12 dark/light cycle (lights on 07:00 h) at 24±1 °C, and given free access to food and water. Before the beginning of the treatments, the animals had a three-day habituation period to the lodging conditions. 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 National Institutes of Health Guide for Care and Use of Laboratory Animals.

4.2. Behavioral procedure

In the first part of our study, we tested the efficacy of the perorally (p.o.) drug intake procedure in producing signs of abstinence 48 h after the interruption of the diazepam regimen, which had lasted for 18 days, by means of the elevated plus-maze test. On day 18, the anxiolytic effects of diazepam were also tested. Two indices of anxiety were used: the percentage of entries and time spent in the open arms of the maze. We also recorded the number of entries in the closed arms as a measure of the animal's motor activity.

4.3. Administration of drugs by oral route

The self-administration of diazepam p.o. in rats suffers the bias of the gustative and temporal factors in function of a delay in the...
production of its reinforcing effects. In this case, water deprivation and sucrose were two elements added to our experimental design that helped us to circumvent this problem. In fact, the use of deprivation for the establishment of motivational drives has been very successful when emotional aspects of the 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. In our study, as we shall see, the maximum dose of 100 μg of sucrose did not have any consequence 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 were changed in these animals, in comparison with the no-treatment group.

The oral voluntary ingestion of diazepam started 3 days after the arrival of the animals at the laboratory’s animal house. The chronic administration of a placebo solution by daily injections can, by itself, induce an increase in the anxiety levels in animals tested in the elevated plus-maze (Griebel et al., 1994). To avoid these caveats we used an oral procedure, adapted by Schleimer et al. (2005), in which the animals were submitted daily to 14 h of water deprivation (19:00 to 9:00), followed by 10 h of water ad libitum (9:00 to 19:00). This procedure began 2 days before the start of the 18 treatment days, as a period of habituation of 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 volume of 1 ml/kg diluted in a solution of 2 ml of tap water added to sucrose (5%) plus propilenoglycol (5%). The solutions were available to the animals, in glass pipettes of 5 ml, at the end of the daily period of water deprivation, delivered during each of the 18 days of treatment. Except for the first 2 days of treatment, in which control and experimental solutions were available for 30 min, the animals that did not drink for 10 min were discarded from the experiments. This was necessary to avoid prolonging the deprivation period. Still, to evaluate the possible aversive effects of water deprivation or reinforcing effects of the sucrose solution per se, a second control group was included in which the animals had food and water ad libitum during the whole period of the experiment. Thus, four groups of animals were formed: a) No treatment group (No Treat, n=20), b) Sucrose p.o. intake (n=21), c) diazepam p.o. intake dependence condition (Dzp DEP, n=16) and d) diazepam p.o. intake withdrawal condition (Dzp W, n=19).

4.4. Elevated plus-maze test

The elevated plus-maze was made of wood and consisted of four arms of equal dimensions (50 cm × 12 cm). Two of the arms were enclosed by 40-cm-high walls and were arranged perpendicularly to two opposite open arms. The apparatus was elevated 50 cm above the floor, with a 1 cm Plexiglas rim surrounding the open arms to prevent falls (Pellow et al., 1985).

The apparatus was located inside an isolated room with 20 lx of luminosity at the end the open arms. The recording of the behaviors of the animals in the plus-maze was made through a camera (Everfocus, USES) linked to a monitor and video cassette, external to the experimental room. The tests were conducted 30 min (we named this situation the dependence 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). The tests lasted 5 min. The animals were placed in the center of the plus-maze facing one of the closed arms. Each animal was tested just once.

4.4.1. Statistical analysis

The data are presented as mean ± S.E.M. The behavioral data were analyzed by means of a one-way ANOVA. The dependent factor was the number of entries in the closed arms and percentage of entries and time spent in the open arms; the independent factor was no treatment (No Treat), sucrose, diazepam dependent (Dzp DEP) or diazepam withdrawal (Dzp W) groups. Newman–Keuls’s 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.

4.5. Fos protein immunoreactivity

We evaluated the Fos-protein expression in rats under 48 h of diazepam-withdrawal. Eleven animals from each of the sucrose and diazepam oral regimens were randomly chosen for immunohistochemical protocols. Two hours after the plus-maze test, these animals were deeply anaesthetized with urethane (1.25 g/kg, i.p., Sigma, USA) and intracardially perfused with 0.1 M phosphate-buffered saline followed by 4% paraformaldehyde in 0.1 M PBS (pH 7.4). The brains were removed and immersed (4 °C) for 2 h in paraformaldehyde and then stored for at least for 48 h in 30% sucrose in 0.1 M PBS cryoprotection. They were then quickly frozen in isopentane (−40 °C) and sliced by the use of a cryostat (−19 °C). To keep the experimental conditions similar for all animals of both sucrose and diazepam-withdrawal groups, all incubations had brain slices of each structure analyzed in this study. Two adjacent series of 40 μm thick brain slices were obtained. One series was Nissl stained and used for neuroanatomical comparison purposes and the other series was collected for the immunohistochemical studies. Tissue sections were collected in 0.1 M PBS and subsequently processed free-floating according to the avidin–biotin procedure, using the Vectastain ABC Elite peroxidase rabbit IgG kit (Vector, USA, ref. PK 6101). All reactions were carried out under agitation at room temperature. The slices were first incubated with 1% H2O2 for 10 min, washed four times with 0.1 M PBS (5 min each) and then incubated overnight at room temperature with the primary Fos rabbit polyclonal IgG (Santa Cruz, USA, SC-52) at a concentration of 1:2000 in PBS+(0.1 M PBS enriched with 0.2% Triton-X and 0.1% Bovine Serum Albumin, BSA). Sections were again washed three times (5 min each) with 0.1 M PBS and incubated for 1 h with secondary Fos biotinylated anti-rabbit IgG (H+L) (Vecstain, Vector Laboratories) at concentration of 1:400 in PBS+. After another series of three 5-min washings in 0.1 M PBS the sections were incubated for 1 h with the avidin–biotin–peroxidase complex in 0.1 M PBS (A and B solution of the kit ABC, Vectastain, Vector Laboratories) at concentration of 1:250 in 0.1 M PBS, and then were again washed three times in 0.1 M PBS (5 min per wash). Fos immunoreactivity was revealed by the addition of the chromogen 3,3′-di-aminobenzidine (DAB, 0.02%, Sigma) to which hydrogen peroxide (0.04%) was added just prior to use. Finally the tissue sections were washed twice with 0.1 M PBS.
4.5.1. Quantification of Fos-positive cells
Tissue sections were mounted on gelatin-coated slides, dehydrated for observation and cell counting under bright-field microscopy. The nomenclature and nuclear boundaries utilized were based on the atlas of Paxinos and Watson (2005). Cells containing a nuclear brown–black reaction product with areas between 10 and 80 μm² were identified and automatically counted as Fos-positive neurons by a computerized image analysis system (Image Pro Plus 4.0, Media Cybernetics, USA). Sections of 26 different regions at different levels in the brain were collected, according to a method used in previous studies (Lamprea et al., 2002; Vianna et al., 2003; Ferreira-Netto et al., 2005). Mounted sections of the tissue were observed using a light microscope (Olympus BX-50) equipped with a video-camera module (Hamatsu Photonics C2400) and coupled to a computerized image analysis system indicated above. Counting of Fos-positive cells was performed under a ×10 objective at a magnification of ×100 in one field per area encompassing the positive cells was performed under a ×10 objective at a module (Hamatsu Photonics C2400) and coupled to a computing system (Image Pro Plus 4.0, Media Cybernetics, USA). This work was supported by grants from FAPESP (04/02859-0).

Acknowledgment

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nucleus accumbens of rats repeatedly exposed to diazepam or imidazenil. Psychopharmacology 131, 34–39.


