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Pharmacology, Biochemistry and Behavior 69 (2001) 431–438

PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR

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Hippocampus and locus coeruleus activity on rats chronically treated with diazepam

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Received 6 October 2000; received in revised form 22 February 2001; accepted 7 March 2001

Abstract

The neural mechanisms underlying benzodiazepine (BZD) dependence remain equivocal. The present studies tested the hypothesis that similar neural circuitry might be involved in the effects of chronic 7-chloro-1-methyl-5-phenyl-3*H*-1,4-benzodiazepine-2(1*H*)-one, diazepam (DZ, Roche), administration and withdrawal. The results of our study showed an increased hippocampal synaptic plasticity in slices from rats chronically treated with DZ (5 mg/kg/18 days), assessed as a decrease of the threshold in the stimulation rate for long-term potentiation (LTP) elicitation. Rats with the same schedule of DZ administration but without signs of withdrawal behaved similarly to vehicle-treated ones (VEH), in the threshold to induce LTP. Furthermore, the activity of locus coeruleus (LC) norepinephrine (NE) neurons in rats tested 24 h after the last DZ injection showed a significant increase. On the other hand, rats that after chronic DZ administration did not develop signs of withdrawal and exhibited a similar pattern of discharge on LC-NE nucleus compared with their controls. We conclude that chronic DZ administration enhances both hippocampal synaptic plasticity and activity of LC-NE neurons. This neural system could be the biological substrate underlying the behavioral alterations accompanying chronic DZ administration and withdrawal. © 2001 Elsevier Science Inc. All rights reserved.

Keywords: Hippocampus; Synaptic plasticity; Locus coeruleus; Noradrenergic transmission; Withdrawal; Dependence; Benzodiazepine

1. Introduction

The pharmacological actions of benzodiazepines (BZDs) are mediated by a high-affinity site on $GABA_A$ receptor, widely distributed on postsynaptic neurons into the central nervous system (CNS). The binding of BZDs agonists results in facilitation of GABA-operated chloride channels, that is, enhancement of the inhibitory actions by GABA (Lüddens et al., 1995; Rabow et al., 1995; Sieghart, 1995; Smith and Olsen, 1995). BZDs are commonly prescribed for the treatment of anxiety and sleep disorders. However, prolonged treatment may lead to dependence with evident withdrawal syndrome (Petursson, 1994).

It is generally understood that the neurophysiological activity of the mammalian brain is maintained by the balance between inhibitory (such as GABA) and excitatory (such as glutamate) neurotransmission. Indeed, there is close interaction between $GABA_A$ receptors and the *N*-methyl-D-aspartate (NMDA) receptors in the CNS (Chaudieu et al., 1994; Stelzer et al., 1987). For example, kindling induced by $GABA_A$ receptor channel blocker pentylenetetrazol was abolished by NMDA receptor antagonist treatment (Corda et al., 1992; Giorgi et al., 1991). A protective effect of muscimol against NMDA-induced neuronal injury has been reported (Ohkuma et al., 1994). Furthermore, Tsuda et al. (1997a,b) have demonstrated that pentylenetetrazol and BDZ site inverse-agonist DMCM-induced seizures are suppressed by the noncompetitive NMDA receptor antagonist dizocilpine.

Previous studies in rodents have shown that chronic exposure to BDZ develops tolerance and physical dependence (File, 1985; Gonzales et al., 1984). Clinical studies have also demonstrated that long-term use of BZDs often results in tolerance to and dependence on many of the therapeutic actions of BZDs (Rickels et al., 1983; Hallstrom and Lader, 1981). Furthermore, severe withdrawal syndrome has been reported with seizures and even death in humans (Owen and Tyrer, 1983; Petursson and Lader, 1981). Thus, the mechanisms by which BZD tolerance and dependence are mediated have been the focus of much recent interest lately.

The NMDA receptors subtype of glutamate receptors may play a very important role in the use-dependent

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0091-3057/01/$ – see front matter © 2001 Elsevier Science Inc. All rights reserved.
PII: S0091-3057(01)00517-2

plasticity of synapses (Bliss and Collingridge, 1993) and long-term potentiation (LTP) (Harris et al., 1984). It has also been consistently supported that epileptiform seizures and excitotoxicity are caused by the large influx of calcium into the cell as a result of NMDA receptor activation (Dingledine et al., 1986). Furthermore, previous results from our laboratory have demonstrated a close relationship between the development of tolerance to hypolocomotor effects of diazepam (DZ) and an increased hippocampal synaptic plasticity in the rat, which was assessed by a lower threshold to induce LTP on hippocampal dentate gyrus of rats tolerant to DZ (Marín et al., 1996).

The locus coeruleus (LC) is the major noradrenergic nucleus in the pons that influences a wide range of brain and behavioral functions including the sleep–waking cycle (Aston-Jones and Bloom, 1981), neural plasticity (Anlezark et al., 1973; Stanton and Sarvey, 1985; Velley and Cardo, 1982), drug abuse (Koob, 1992; Corrodi et al., 1967), and stress responses (Aston-Jones et al., 1994; Owen and Tyrer, 1983). In addition, this system has been proposed to modulate several cognitive functions including learning and attention involved in arousal and vigilance (Velley and Cardo, 1982; Aston-Jones et al., 1991; Robins and Everitt, 1995). Its activation is one of the major promoters of the physical withdrawal symptoms for different types of psychoactive drugs, involving autonomic and spinal cord function (Koob, 1992). Clinical and laboratory reports on BZD withdrawal describe symptoms such as anxiety, insomnia, and other hyperexcitability phenomena, which suggest enhanced adrenergic activation. Biochemical studies in both humans and animals showed that acute and long-term use of BZD was associated with decreased levels of norepinephrine (NE) and its metabolites, while BZD withdrawal was associated with increased NE levels (Nestler et al., 1993, 1994).

The present studies tested the hypothesis whether similar neural systems are recruited during DZ tolerance and withdrawal, or associated with a phenomenon involving hippocampal synaptic transmission. Furthermore, and considering that the role of LC, amygdala, and the nucleus raphe (Christie et al., 1997) in the withdrawal syndrome of DZ and other psychoactive drugs is not clear, and that different cellular mechanisms might be involved, we decided to look into the participation of LC noradrenergic activity in the effects of chronic DZ administration during withdrawal.

2. Material and methods

2.1. Animals

In all, 59 male Wistar rats 60–75 days old and weighing 190–260 g were used. Animals were housed in groups of five in their home boxes and kept under a 12:12 L/D cycle (light on at 7 a.m.) and regular temperature conditions ($22 \pm 1^{\circ}C$). Food and water were available ad lib.

2.2. Elevated plus-maze apparatus

The plus-maze consisted of two open arms, 50×10 cm, and two enclosed arms, $50 \times 10 \times 40$ cm, with an open roof, arranged so that the two open arms were opposite each other. The arms extended from a central platform 10×10 cm. The maze was elevated to a height of 50 cm. The measures indicated were taken by an observer sitting in the same room as reported by Pellow and File (1986). The number of closed arm entries represents a measure of locomotor activity (Cruz et al., 1994).

2.3. Procedure

Animals were injected daily with either DZ (5 mg/kg) or vehicle (VEH, distilled water with a drop of Tween 80 and propyleneglycol 5%) during 18 days.

Clinical experiments have shown that anxiety, muscle spasms, and seizures are major withdrawal signs after discontinuation of chronic BZD treatment (Woods et al., 1987, 1992). On the basis of these criteria we selected our experimental groups taking into account the activity of the rats in an elevated plus-maze. Accordingly, 24 h after the last injection, each animal was individually placed in the elevated plus-maze apparatus for a 5-min period, during which anxiety was assessed by the percentage of time spent in the open arms. Depending on the activity of the animals, we made designed three groups: animals that showed anxiety signs were dubbed "DZ-dependent group" (DZ-D), those that received DZ but did not show anxiety signs were called "DZ nondependent group" (DZ-ND), and finally, animals that received VEH injection (control group). Also, to discard any influence of the plus-maze exposure to the synaptic plasticity on hippocampus electrophysiological experiments (see below), another group was treated with the same administration schedule (DZ or VEH), but not exposed to the plus-maze test. Immediately after the plus-maze test, animals were sedated with a 50:50 mixture of CO_2/O_2 and sacrificed by cervical dislocation for hippocampal electrophysiological assays.

In order to evaluate the effect of chronic DZ treatment, another group was injected daily with either DZ 5 mg/kg or VEH during 18 days. Considering the brain half-life of DZ (0.89 h) these animals were sacrificed 4 h after the last injection in order to avoid withdrawal (Friedman et al., 1986; Davis and Gallager, 1988).

These conditions meet the standards for care of laboratory animals as outlined in the NIH Guide for the Care and Use of Laboratory Animals (1996).

2.4. Electrophysiological procedures

2.4.1. Hippocampus

The subjects were rats from the groups previously described (see Procedure section). The hippocampal slices in electrophysiological experiments were obtained from

animals sacrificed 24 h after the last DZ or VEH injection (DZ-D, DZ-ND, and VEH groups) or 4 h after the last DZ administration.

Electrophysiological experiments were done using the in vitro hippocampal slice preparation described elsewhere by Ramirez et al. (1988). Briefly, rats were sacrificed between 11:00 a.m. and 12:00 noon to prevent variations caused by circadian rhythms or nonspecific stressors (Tayler and Di Scenna, 1987). The hippocampal formation was dissected and transverse slices, approximately 400 μm thick, were placed in a recording chamber, perfused with standard solution saturated with 95% O_2 and 5% CO_2. Rate of perfusion was 2–3 ml/min, while the bathing solution temperature was kept at 28°C. A stimulating electrode was placed in the perforant path (PP) and a recording microelectrode was inserted in the dentate granule cell body layer. Only slices showing a healthy response were included in this electrophysiological study. Ten field potentials that responded to the stimuli were sampled at 0.2 Hz, averaged on line using a PC computer, and the data thus obtained were stored in diskettes for further analysis. Once no further changes were observed in the amplitude of the response, which included population spike (PS) for 20 min, the intensity of the electrical stimulus to the PP was set at the value that would elicit spikes approximately 30% of the maximum response. The LTP eliciting frequency threshold was determined as described by Ramirez et al. (1988). Tetanus consisting of a train of pulses (0.5 ms) of 2-s duration and with increasing frequency was delivered to the slice at intervals that ranged from 20 min up to 45 min, starting with a 5-Hz tetanus, whose intensity increased with each train to 10, 25, 50, 75, 100, 150 up to 200 Hz. At 15 to 20 min after a tetanus, a new averaged response was recorded; when LTP was not observed, another tetanus at the next higher frequency was applied. LTP was considered to have occurred when the amplitude of the evoked PS (Fig. 2) recorded after the tetanus had risen by at least 30% and persisted from 20 min to 1 h. Once LTP was achieved, no further tetanus were given.

2.4.2. Extracellular single-cell recordings on LC neurons

Animals from VEH, DZ-D, and DZ-ND groups, described in the Procedure section, were used for extracellular single-unit recordings as follows: rats were anesthetized with chloral hydrate (400 mg/kg ip) and supplementary doses of anesthetics were administered through a dorsal tail vein when needed, to maintain surgical anesthesia throughout the experiment. These conditions meet or exceed the standards for the care of laboratory animals as outlined in the NIH Guide for the Care and Use of Laboratory Animals. Techniques used for extracellular single-cell recording have been described in detail elsewhere (Ramirez and Wang, 1986a,b; Pavcovich et al., 1990). Briefly, rats were mounted on to a stereotaxic frame, their skulls were exposed, and a hole was drilled above the LC, where an electrode was lowered (1.1–1.3 mm posterior to lambda, 1.1–1.3 mm lateral to midline suture, and 5.5–6.5 mm below the dura; atlas of Paxinos and Watson, 1986) by means of a hydraulic microdrive. The number of spontaneously active cells per track (five-track average per animal) and their firing rate were assessed. The firing rate was obtained from the counted cells that displayed a signal-to-noise ratio of 2:1 or more. LC noradrenergic neurons displayed the following characteristics: (a) positive–negative action potentials lasting approximately 2 ms, often with a notch between the initial segment and the somatodendritic spike component; (b) a firing rate of 0.5–3.0 spike/s; (c) burst of firing followed by a quiescent period in response to pinching of the contralateral paw (Cedarbaum and Aghajanian, 1977). These properties fulfil electrophysiological criteria for the identification of LC noradrenergic cells (Aghajanian et al., 1977; Ramirez and Wang, 1986a,b; Pavcovich et al., 1990). Electrode potentials, which had been previously passed through a high-impedance amplifier, were displayed on an oscilloscope. The electrical signals were passed through a window discriminator and screened on an audio amplifier. Upon completion of the experiments, the cell location was marked by passing a 25-mA cathodal current through the recording electrode for 15 min and a spot of Fast green dye was deposited. Rats were then perfused with phosphate-buffered 10% formaline solution. Serial frozen sections, 50-μm thick, were sliced and the dye spot was microscopically traced.

2.5. Statistics

All experimental data were analyzed by one-way ANOVA, followed by Newman–Keuls pairwise compari-

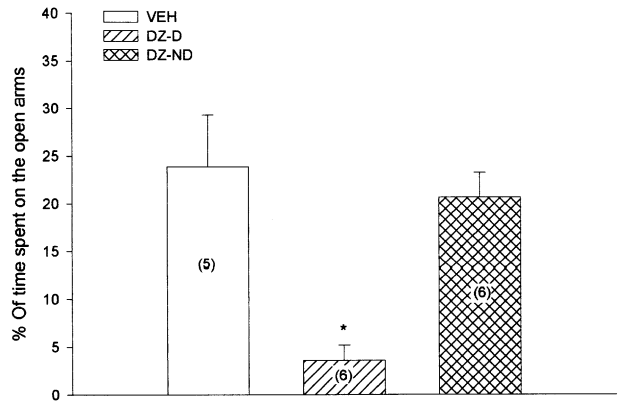

Fig. 1. Mean (±S.E.M.) percentage of time (s) spent in the open arms in rats given a 5-min test in the elevated plus-maze, 24 h after the last DZ or VEH administration. Bars represent the mean and vertical bars the S.E.M. The number of animals is indicated in parentheses. * $P<.002$, significantly different from VEH and DZ-ND groups.

Table 1
Mean (±S.E.M.) number of arm entries made by rats during a 5-min test in the elevated plus-maze

Groups (number of animals)	Open	Close	Totals
VEH (5)	3.4±1.0	8.60±1.5	12.0±2.1
DZ-D (6)	0.7±0.2*	7.7±0.7	8.3±0.5
DZ-ND (6)	3.0±0.7	5.7±0.8	8.7±1.1

* $P<.03$ significantly different from VEH and DZ-ND groups. $F(2,14)=5.73$.

sons of means. $P\leq.05$ represents a significant difference between groups.

3. Results

Fig. 1 and Table 1 show the time spent exploring the open arms and the number of entries in each arm, in a novel elevated plus-maze for the rats, 24 h after the discontinuation of chronic administration of DZ (5 mg/kg/day) for 18 days or VEH. The rats that spent less time exploring the open arms of the plus-maze were included in the group dubbed DZ-D (3.6±1.6). There was another group of rats spending similar time as controls (23.9±5.4), which was DZ-ND group (20.7±2.6) $F(2,14)=13.9$, $P<.0005$. Rats from the DZ-ND exhibited no sign of anxiety, in spite of the chronic 18 days DZ administration (5 mg/kg). Furthermore, there were no differences in the locomotor activity assessed by the number of closed arm entries among the different groups (Table 1).

Fig. 2 (insert) shows the characteristic evoked field response in the granule cell layer of the dentate gyrus after single-pulse stimulation in the PP. It consisted of a gradual positive-going field excitatory postsynaptic potential (EPSP) with a sharp negative-going PS superimposed on the rising phase of the EPSP. The EPSP reflects synaptic currents at PP-dentate granule cell synapses in stratum moleculare, whereas the PS reflects the synchronous action potential discharge of granule cell bodies in stratum granulosum. This panel shows the increased amplitude of PS after an effective tetanus.

In the same figure, we can see the threshold necessary to induce LTP, measured in hertz, in DZ-D, DZ-ND, and VEH groups. Rats chronically treated with DZ for 18 days, with anxiety signs, showed an increased susceptibility to generate LTP on hippocampal dentate gyrus (DZ-D: 33.3±3.65), compared with controls (VEH: 64.00±10.95), $P<.03$. Furthermore, rats from DZ-ND group exhibited similar susceptibility to induce LTP on hippocampus (83.33±11.89) as VEH group, $F(2,14)=9.21$.

The increased sensitivity to generate LTP noticed 24 h after discontinuation of chronic DZ on the DZ-D, may be partially due to the withdrawal phenomenon or consequence of chronic DZ administration. To assess this possibility, we examined the sensitivity of LTP induction on hippocampal dentate gyrus on rats sacrificed 4 h after the last injection of

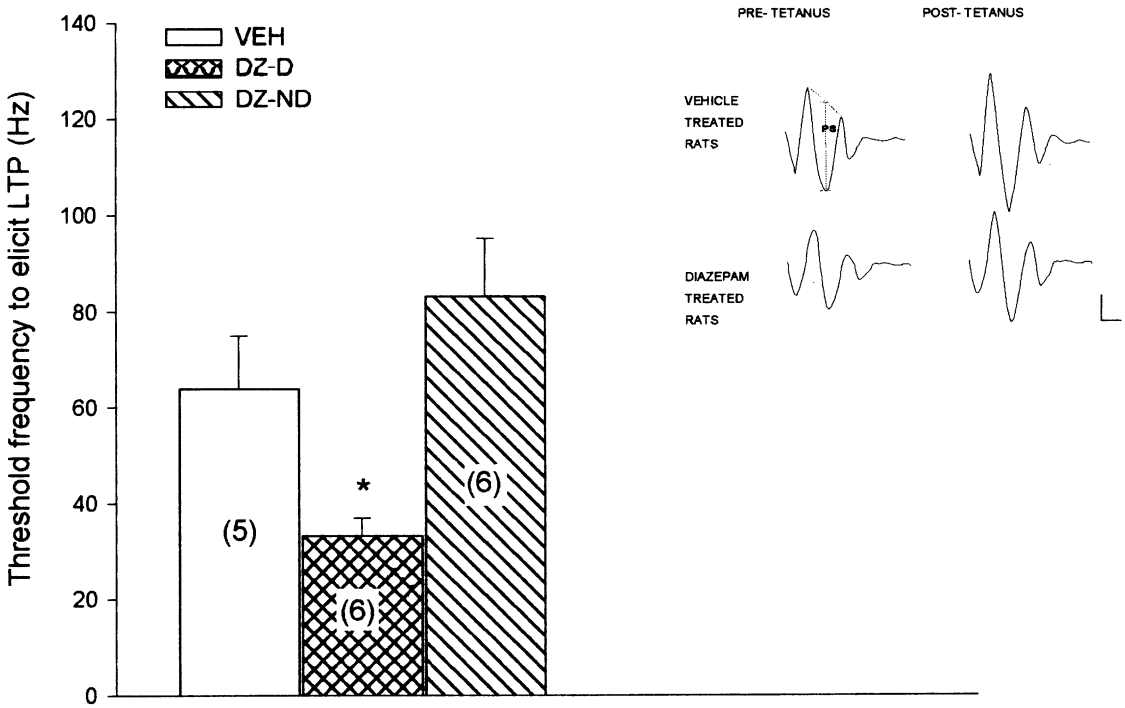

Fig. 2. Threshold frequency to elicit LTP in hippocampal slices from DZ-D, DZ-ND, and VEH animals, sacrificed 24 h after the last DZ or VEH administration. Bars represent the mean and vertical bars the S.E.M. The number of animals is indicated in parentheses. * $P<.03$, significantly different from VEH and DZ-ND groups. Insert, an example of field potential of responses from granule cells layer of the dentate gyrus evoked by stimulation of the PP in a hippocampal slice. Pretetanus was delivered at 0.2 Hz; posttetanus shows the responses recorded after a train of high-frequency stimulation. Calibration bars represent 5 ms and 0.5 mV. PS, population spike.

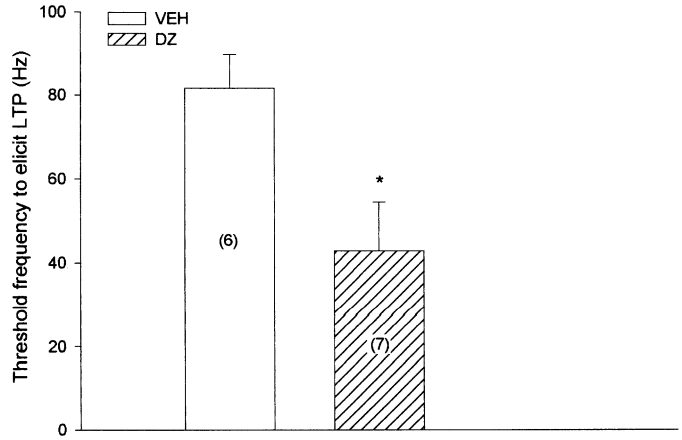

Fig. 3. Threshold frequency to elicit LTP in hippocampal slices from animals treated with VEH or DZ during 18 days and sacrificed 4 h after the last DZ or VEH administration, without plus-maze experience. Bars represent the mean and vertical bars the S.E.M. The number of animals is indicated in parentheses. * $P < .02$, significantly different from VEH-administered group.

chronic DZ administration, time during which animals did not develop withdrawal anxiety signs. Fig. 3 shows an increased sensitivity to induce LTP in these animals (42.86 ± 11.72) as compared with controls (81.67 ± 8.21), $F(1,11) = 8.08$, $P < .016$. This increased sensitivity to generate LTP was similar to that observed in animals sacrificed 24 h after the last chronic DZ administration (DZ-D group), when they experienced withdrawal signs.

In order to demonstrate that the previous exposure to elevated plus-maze did not influence the sensitivity to induce LTP on these animals, a group of rats were sacrificed 24 h after chronic DZ (nine animals) or VEH (five animals) without previous exposure to plus-maze test. We found no differences between DZ chronic animals (51.6 ± 11.5) and those previously exposed to plus-maze test (DZ-D: 33.3 ± 3.65) in the threshold to induce LTP.

To study the role of the LC nucleus on the effects of chronic DZ administration, the activity of LC-NE neurons was evaluated on DZ-D, DZ-ND, and VEH rats, using the extracellular single-unit recording method. In Table 2, we can see that rats showing increased anxiety on an elevated plus-maze 24 h after the last injection of chronic DZ administration (DZ-D), exhibited an enhanced neuronal activity on LC-NE neurons, which was assessed by the increased number of spontaneous cells per track and its firing rate, compared with control group (VEH). On the other hand, rats showing no withdrawal signs (DZ-ND) such as anxiety evidenced a normal pattern of LC electrical activity.

Table 2
Effects produced on the number of spontaneously active LC cells (cells per track) and their average firing rate (spikes/s) during DZ withdrawal syndrome

Groups (number of animals)	Cells per track	Firing rate (number of cells)
VEH (5)	4.45 ± 0.15	1.67 ± 0.16 (11)
DZ-D (5)	6.67 ± 0.70**	3.13 ± 0.31* (14)
DZ-ND (5)	4.57 ± 0.33	1.44 ± 0.13 (13)

* $P < .001$.
** $P < .05$ significantly different from VEH and DZ-ND groups.

4. Discussion

The NMDA receptor has been claimed to play a role in the mechanisms of tolerance to and dependence on BZD (Tsuda et al., 1998) and other psychoactive drugs such as opioids, alcohol, and barbiturates (Zhu et al., 1999; Oh et al., 1998; Jang et al., 1999; Calton et al., 1999). Furthermore, different areas of the brain have been involved in the expression of withdrawal, for instance, LC, amygdala, and raphe nucleus (Christie et al., 1997). The major finding of the present study is the significant increase in hippocampal synaptic plasticity during withdrawal following discontinuation of chronic DZ administration. Similar increased hippocampal synaptic plasticity noticed in chronically DZ-treated rats showed no physical withdrawal, such as anxiety in an elevated plus-maze.

Previous findings from our laboratory have demonstrated a positive correlation between the development of tolerance to DZ (5 mg/kg/day) along 4 days and the increased hippocampal synaptic plasticity (Marín et al., 1996). Considering the results of the present investigation, it seems likely that a similar plastic phenomenon may occur on hippocampal formation, after chronic DZ administration and withdrawal. The increased hippocampal synaptic plasticity could be due to an increased glutamatergic transmission on the hippocampal dentate gyrus. In fact, we cannot rule out a decreased GABAergic input on this brain area. Furthermore, it seems that previous exposure to an elevated plus-maze has no influence on the increased hippocampal synaptic plasticity observed after chronic DZ treatment or during withdrawal, since animals unexposed to

plus-maze showed a similar threshold to induce LTP as the control and treated groups. The participation of the NMDA receptor in the generation of LTP has been largely demonstrated (Bliss and Collingridge, 1993; Harris et al., 1984). On the other hand, antagonists to these receptors or channel blockade associated to these receptors are able to impair the LTP generations on hippocampal formation (Collingridge and Bliss, 1995) and, ameliorate the behavioral expression of physical signs of dependence to BZD and other different psychoactive drugs (Trujillo and Akil, 1991; Rabbani et al., 1994). It is possible that a plastic phenomenon, such as hippocampal LTP, be the underlying biological substrate to the behavioral effects of chronic DZ administration and withdrawal symptoms.

The participation of the NA system in promoting a full withdrawal syndrome after long-term use of BDZ has been well established in many studies (Goudie et al., 1993; Kunchandy and Kulkarni, 1986; Rasmussen et al., 1994; Corrodi et al., 1971; Rastogi et al., 1977, 1978). Besides, drugs with a strong direct effect on LC-NE activity have a higher potential for tolerance and withdrawal effects (Redmon, 1987). In our study, rats chronically treated with DZ for 18 days displayed an increased LC-NE electrical activity, which was assessed by an augmented number of spontaneously active cells per track, and their firing rate when compared with rats treated with VEH. Interestingly, DZ-treated rats without physical signs of withdrawal, such as increased anxiety in an elevated plus-maze, showed no differences in their LC electrical activity as compared with controls. The enhancement of LC-NE activity, observed after discontinuation of DZ chronic administration, may account for both, the physical withdrawal symptoms involving autonomic function and the increased hippocampal synaptic plasticity observed on withdrawal, since it has been demonstrated on hippocampal slices of adult rats, that the perfusion of adrenergic agonists promotes lasting synaptic plasticity in the adult CNS (Izumi and Zorumski, 1999).

BZD withdrawal symptoms may be an heterogeneous phenomenon involving a number of different underlying signs mediate by different mechanisms (Goudie and Leathley, 1990, 1991, 1995). In the current research, we have used the increase in anxiety as a behavioral expression of a mild symptom of discontinuation of DZ chronic administration, which was assessed by the activity of the rat in an elevated plus-maze. Under our experimental conditions an increased hippocampal synaptic plasticity was found after chronic DZ administration (18 days) and withdrawal. The increased plasticity agrees with the enhanced LC-NE activity during withdrawal but not after chronic DZ treatment.

In short, our results confirm the potential role of the hippocampus glutamatergic and GABAergic transmission in the mechanisms underlying the altered behavior characterizing DZ withdrawal. The LC-NE system augmented activity reported here is in agreement with previous findings that have demonstrated increased NA levels and metabolites in both human and rodent DZ withdrawal. Furthermore, it has been proposed that the DZ-increased LC-NE activity affects the regulation of NA release indirectly through GABA, a primary inhibiting neurotransmitter system of the brain (Duka et al., 1986; Nutt and Molyneux, 1987; Suzdak and Gianutsos, 1985). The medial prefrontal cortex provides a powerful excitatory influence on LC neurons, which is mediated by excitatory amino acid inputs (Jodo and Aston-Jones, 1997). These findings indicate a source whereby cognitive processing or emotional activity influences LC function, and reinforces the idea that LC is fully involved in modulation of higher mental activity. In the light of this finding, we can also speculate that the increased LC-NE activity can be a consequence of the cortical activation produced by behavioral alterations observed during withdrawal. Another aspect of our results is the fact that the hyper LC-NE activity observed only after the presence of increased anxiety validates this as further evidence of DZ withdrawal. We cannot rule out the participation of other brain areas and cellular mechanisms in the behavioral alterations characterizing withdrawal symptoms.

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