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Increased Fear Learning Coincides with Neuronal Dysinhibition and Facilitated LTP in the Basolateral Amygdala following Benzodiazepine Withdrawal in Rats

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Animals chronically administered with diazepam (DZM – 2 mg/kg/day i.p.) or vehicle (VEH) for 21 days were tested in a fear-conditioning paradigm 4 days after the last administration. Increased freezing was observed in DZM withdrawn rats as compared to VEH injected control rats in both associative and nonassociative context and this increase was greatest for the DZM withdrawal group in the paired context. In animals anesthetized with urethane, single pulses in the medial prefrontal cortex evoked a field potential including a population spike (PS) in the basolateral complex of the amygdala (BLA) of control animals, whereas in DZM withdrawn animals multiple PSs were evoked. In brain slices from control rats, stimulation of the external capsule evoked a field potential including a PS in the BLA, whereas in DZM withdrawn rats multiple PSs were evoked. The amplitude of the PS was smaller in slices obtained from DZM withdrawn rats than from control rats, and paired pulse inhibition was significantly less in the former. Perfusion with DZM 2 μM of slices obtained from DZM withdrawn rats eliminated repetitive spiking. GABAergic blockade with 50 μM picrotoxin in control rats resulted in the appearance of multiple secondary PSs. In slices from DZM withdrawn rats high-frequency stimulation induced a highly significant potentiation that lasted at least 2 h (LTP), whereas in control rats the same stimulation did not induce LTP. Neuronal hyperexcitability leading to facilitated LTP observed in BLA of DZM withdrawn rats could be due to depressed GABAergic activity (dysinhibition). The increased synaptic plasticity may be at the root of the increased fear learning observed in withdrawn animals.
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INTRODUCTION

Benzodiazepines (BDZ) are frequently prescribed for the treatment of anxiety and sleep disorders. The main problem with long-term BDZ usage lies in the development of tolerance and dependence (Petursson and Lader, 1981; Greenblatt and Shader, 1978; Schweizer and Rickels, 1998). In fact, numerous clinical and experimental reports have shown that the abrupt discontinuation from repeated BDZ administration leads to a well-recognized withdrawal syndrome (File, 1990; Schweizer and Rickels, 1998; Bateson, 2002). In laboratory animals, discontinuation from chronic treatment with different BDZ agents resulted in spontaneous seizures, enhanced sensitivity to audiogenic seizures, increased anxiety, tremors, and weight loss, among other effects (Martin *et al*, 1993; Boisse *et al*, 1986; Emmett *et al*, 1983; Ryan and Boisse, 1983; File, 1990). Studies conducted to evaluate behavioral and neurochemical responses to diverse stressful situations demonstrated disturbances in behavioral reactivity and altered $GABA_A$ receptor functioning, in animals previously subjected to BDZ discontinuation and later on exposed to novel aversive stimuli (Martijena *et al*, 1996, 1997; Lacerra *et al*, 1999). Hence, brief exposure to mild aversive stimulation that would induce minimal behavioral and emotional sequelae in normal animals induced exaggerated emotional responses and stress-induced behavioral deficits lasting several days after the interruption of chronic BDZ. Based on such findings we have previously proposed (Martijena *et al*, 1996) that the long-lasting occurrence of exaggerated emotional reactions to mild aversive stimulation could be an additional component of BDZ withdrawal. Therefore, previous experience with abrupt BDZ discontinuation could critically influence the way withdrawn individuals react to further environmental challenges.

Classic fear conditioning is one of the most widely used paradigms to evaluate associative emotional learning. In this aversive learning paradigm, a neutral conditioned

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stimulus (CS) is repeatedly paired with an unpleasant and unconditioned stimulus (US) such as an electric footshock. As a result of such pairing of events, the previously neutral CS then produces a hypothetical state of fear that is expressed as freezing, one of the most prominent behavioral signs of fear in rats (Blanchard and Blanchard, 1971, 1969; Fendt and Fanselow, 1999). In the current study, in order to evaluate the influence of BDZ withdrawal on subsequent emotional learning, BDZ withdrawn animals were tested in a fear-conditioning task.

A substantial amount of evidence supports the notion that the amygdala is a key brain structure in the modulation of emotional behavior (Davis, 1992; LeDoux, 2000; Davis, 1997; Fendt and Fanselow, 1999; Maren, 1999) and particularly in pavlovian aversive learning (LeDoux *et al*, 1990; Gewirtz and Davis, 1997; Miserendino *et al*, 1990; Fanselow and Kim, 1994). It is known that the basolateral complex (BLA) receives relevant information from the environment via hippocampal, thalamic, and cortical afferents (McDonald, 1998; Davis and Whalen, 2001). In turn, the BLA projects to the central nucleus of the amygdala, which then projects to midbrain and brainstem areas involved in the coordination of endocrine, somatic, and behavioral responses to aversive stimulation characteristic of fear and anxiety (Charney *et al*, 1998; Fendt and Fanselow, 1999; Davis and Whalen, 2001). Considering that a prevailing view supports a critical role of the BLA in associative aversive learning as well as in fearful response to unconditioned aversive stimuli (Davis, 1997; Vazdarjanova *et al*, 2001), we also studied the effects of BDZ withdrawal on the synaptic responses in the BLA, in order to relate the behavioral effect of BDZ withdrawal with potential differences in synaptic transmission in this brain region. To this end, we recorded the potentials evoked in BLA by stimulation of the medial prefrontal cortex (mPFC) in DZM withdrawn rats *in vivo*. Also, in slices of the BLA obtained from DZM withdrawn animals stimuli were applied to cortical afferents to the BLA contained in the external capsule (EC) (De Olmos *et al*, 1985; Fisk and Wyss, 2000). A growing amount of evidence indicates that the synaptic modifications in the BLA, which accompany behavioral learned fear, are mechanistically similar to long-term potentiation (LTP) evoked by electric stimulation in amygdala slices (Shumyatsky *et al*, 2002). Given that *in vitro* and *in vivo* studies have strongly suggested that the BLA could be a locus of synaptic neuroplasticity – such as LTP – and that this process is required to store pavlovian aversive learning (McKernan and Shinnick-Gallagher, 1997; Quirk *et al*, 1995; Rogan and LeDoux, 1995; Fanselow and Ledoux, 1999; Maren, 1999; LeDoux, 2000; Doyère *et al*, 2003), we also evaluated whether prior BDZ withdrawal affects the induction of LTP in the BLA following high-frequency stimulation (HFS) of the EC.

METHODS

Animals

Wistar male rats weighing 250–280 g were used in this study. Animals were housed in standard laboratory Plexiglas cages (four per cage) at the animal colony of the Department of Pharmacology of the Facultad de Ciencias Químicas, Universidad Nacional de Córdoba. A 12 h day–night cycle (lights on at 0700) was maintained throughout the experiment and all test procedures were performed during the light cycle, between 1000 and 1600. Food and water were provided *ad libitum* throughout the study. The protocols used were approved by the Animal Care Committee of the Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, consistent with the standards for the care and use of laboratory animals as outlined in the NIH Guide for the Care and Use of Laboratory animals.

Chronic Diazepam Treatment

Diazepam (DZM) was chronically administered as previously described (File, 1990; Martijena *et al*, 1996). Diazepam was ultrasonically dispersed in a vehicle (VEH) consisting of 1 drop of Tween 80 per 2 ml of distilled water. The control group received the VEH only (Tween 80 + distilled water). All animals were daily weighed and handled and received a daily injection of DZM (2 mg/kg i.p.) or VEH for 21 days. All injections were given in a volume of 1 ml/kg. Interruption of this chronic schedule has been extensively reported to induce behavioral changes characteristic of DZM withdrawal without any signs of spontaneous seizures (File and Andrews, 1991; Lacerra *et al*, 1999; Martijena *et al*, 1996, 2001).

Behavior

Behavioral apparatus. Chambers in separate rooms were used for the experiment designed to evaluate fear conditioning. These chambers were different in size, location, color, illumination, floor, and walls. The first chamber, designated as context A, was made of gray plastic ($20 \times 23 \times 20$ cm) with a clear lid. The floor consisted of 10 steel grid bars spaced 1.0 cm apart. Illumination was provided by a 2.5 W white lightbulb. A shock generator was attached to context A to provide footshock through the grid floor. The second chamber, designated as context B, was made of wood and had a transparent plastic lid, black walls, and black rubber floor; dimensions being $33 \times 25 \times 33$ cm. This procedure was meant to keep testing chamber B as different as possible from the one originally used during training (context A), and hence maximize the possibility of obtaining differential levels of expression of the acquired memory. Obviously, differences in the expression of learning in the present circumstances might obey to contextual changes and/or changes regarding discrete stimuli that define the context. Both chambers were cleaned before and after use with a 5% ammonium hydroxide solution. For the testing phase, context A was referred to as the paired context and context B was referred to as the unpaired context.

Behavioral procedures. Subjects were 48 adult rats. Half the rats were randomly assigned to chronic VEH and the other half to chronic DZM. At 4 days after the last injection all animals were examined in the fear-conditioning paradigm. On the day of conditioning or testing, rats were transported from the colony room to an adjacent acoustically isolated room, where they remained in their cages during the

running of the conditioning or the testing experiment. Different acoustically isolated rooms were used to run the training and the testing phases. During the conditioning session, rats were individually placed in context A and allowed a 3 min acclimation period (preshock period) and then given five scrambled footshocks sessions (0.5 mA, 3 s duration and intershock interval 30 s; unconditioned stimuli). They remained in the chamber for an additional 2 min (postshock period), and after this period rats were put in their home cages and returned to the colony room. All testing for fear conditioning was performed 24 h after conditioning, rats from each group (VEH treated and DZM withdrawn animals) were randomly assigned into two subgroups: half of the animals was reintroduced in context A (paired context) for a 5 min period (without shocks) and the other half exposed for a 5 min period to context B (unpaired context). Freezing behavior, commonly used as an index of fear in rats (Blanchard and Blanchard, 1969), was video recorded during the 3 min preshock period, the 2 min postshock period and during the entire 5 min testing period in context A or in context B.

Crouching without body movement – except that associated with breathing – was considered as freezing behavior. A person blind with regard to the experimental condition of each animal scored the behavior displayed by the animals. The measure of fear was quantified as the amount of time spent freezing during the pre- and postshock periods; results were analyzed by one-way ANOVA. Time spent freezing during the test period in context A or B was analyzed by two-way ANOVA. *Post hoc* comparisons were performed using the Newman–Keuls test. A p-value of 0.05 or less was regarded as indicating a significant difference between groups.

Electrophysiology

In vivo *recording of evoked potentials*. Control and withdrawn animals were anesthetized with urethane (1 mg/kg b.w., i.p.) and placed in a stereotaxic frame 4 days after the last administration of DZM or VEH. After cutting the skin over the skull, a craniotomy was made to allow the penetration of recording and stimulating electrodes. The recording electrode directed to the BLA was a glass pipette filled with 2% Pontamine Sky Blue in 2 M NaCl (0.5–2 MΩ) connected to a differential AC amplifier with a pass band of 1 Hz to 1 kHz. Stereotaxic parameters for the BLA were AP −3.14, V −8.7, and L 5 according to the atlas of Paxinos and Watson (1997). The amplifier output was monitored on a digital oscilloscope and sampled at a rate of 2 kHz with a TL-1/DMA interface board for storage on a PC hard disk using the pCLAMP software for experimental control, data acquisition, and analysis. The stimulation electrode directed to the mPFC was constructed with PtIr wire 50 μm in diameter isolated with Teflon except for the cut ends; stereotaxic coordinates were AP +2.7, V −4.3, and L 0.7. Stimulation consisted of monophasic square pulses (0.2 ms at 0.06 Hz) delivered by a pulse generator with constant current isolation unit. Graded stimulus intensities were used to evaluate the evoked response from threshold to maximal amplitude; 16 records were obtained for each stimulus intensity and averaged offline. At the termination of the experiment, 1 mA of anodic current was passed through the stimulating electrode during 20–30 s as well as anodic square pulses of 200 ms at 1 Hz during 30 min to mark the site of stimulation and recording, respectively. After the animal was killed with ether, the brain was fixed with formalin and subsequently cut serially in a freezing microtome. For exact placement of recording and stimulation sites, the unstained serial sections were studied as described by Guzman-Flores *et al* (1958) and plotted on the plates of the atlas; only animals with correctly placed electrodes were included in the results.

In vitro *recording of evoked potentials*. Control and withdrawn animals without any further manipulation were killed by decapitation 4 days after the last administration of DZM or VEH. Their brains were quickly removed and placed in ice-cold (−3°C) oxygenated artificial cerebrospinal fluid (ACSF) consisting of (in mM) 124 NaCl, 2.60 KCl, 1.25 KH_2PO_4, 2 Mg_2SO_4, 26 $NaHCO_3$, 2 CaCl, and 10 glucose, bubbled with a 95% O_2 −5% CO_2 mixture and adjusted to a final pH of 7.36–7.4 with NaOH 1 N. Working on an ice block, the brain was divided sagitally 2 mm from the midsagital plane and laid on the sagital face created by this cut. An oblique cut parallel to the olfactory bundle was made behind the temporal lobe; this cutting plane was adopted so as to increase the preservation of incoming fibers from the cortex reaching the BLA following a descending oblique path (Fisk and Wyss, 2000). The anterior part of the brain laying on the surface created by this cut was glued with cyanoacrylate to the cutting block of a vibratome. Oblique frontal slices 400 μm thick containing the amygdala and EC were cut and stored for 1 h in oxygenated ACSF maintained at room temperature. For recording, slices were transferred to a chamber continuously perfused with oxygenated ACSF (2 ml/min) maintained at 30 ± 1°C. Recordings were obtained from the BLA, with stimulation applied to the EC (Figure 3a). Recording and stimulation conditions were as described above for experiments *in vivo*.

In order to evaluate the response of BLA neurons to cortical afferent input, the amplitude of the response to increasing stimulus intensity was recorded. The population spike (PS, see Results) amplitude was measured as the distance from the maximal negative-going peak of the PS to a line tangent to the lower and upper shoulders (Figure 2a), based on the average of 16 responses per point. At least four stimulus intensities (0.2–7 mA) were applied in every case, covering the range from threshold to supramaximal intensity; the results were used to plot I/O curves for the amplitude of the PS. The magnitude of recurrent inhibition was assessed by comparing the height of PSs generated by double pulse stimulation to the EC. The interval between double pulses (20, 40, 60, 100 ms) was adjusted to fall within the time course of the intracellularly recorded inhibitory postsynaptic potential (IPSP) attributed to activation of GABAergic interneurons (Rainnie *et al*, 1991b). In these experiments, the intensity of stimulation selected was the one that would evoke a potential corresponding to 50% of maximal response. The variation in response amplitude to the second stimulus was normalized to the amplitude of the first response. PSs were counted by visual inspection of individual records obtained at maximal stimulation and

averaged for each case. To induce LTP a single train of 1 s duration at 100 Hz was administered, selecting the stimulation intensity that evoked 50% of maximum PS response. To evaluate the potentiation, recordings were repeated 10, 20, 40, 60, 80, 100, and 120 min after HFS. For each slice the raw data for the PS amplitude was expressed as percentage of the control records obtained before HFS. For statistical purposes, percentages were normalized by logarithmic transformation and compared using ANOVA followed by Tukey's (HSD) *post hoc* test.

Drugs were applied to the slice by switching the bath superfusate from normal ACSF to ACSF containing Diazepam (Roche) 2 μM, Picrotoxin 50 μM (PTX), $CdCl_2$ 100 μM, 6-cyano-7-nitroquinoxaline-2,3-dione 30 μM (CNQX), or D-2-amino-5-phosphonopentanoic acid 10 μM (APV). Except for diazepam, all drugs were obtained from Sigma-Aldrich Co.

RESULTS

Behavioural Experiments

Effect of DZM withdrawal on fear learning. Figure 1 shows the effect of DZM withdrawal on the freezing behavior evaluated 24 h after footshock, in the context paired with shock (context A) or in a novel unpaired context (context B). As can be seen, DZM withdrawn rats, regardless of test condition, display more freezing than VEH rats (ANOVA showed a significant effect of pretreatment ($F(1, 44) = 35.88$; $p = 0.000001$)). In addition, all animals tested in the paired context, regardless of drug pretreatment, display more time freezing than animals tested in the unpaired context (ANOVA showed a significant effect of test condition ($F(1, 44) = 27.59$; $p = 0.0000001$)). Moreover, the increase in the amount of freezing in the paired environment as compared to the unpaired environment was greater in the DZM withdrawal group than in the VEH group, suggesting

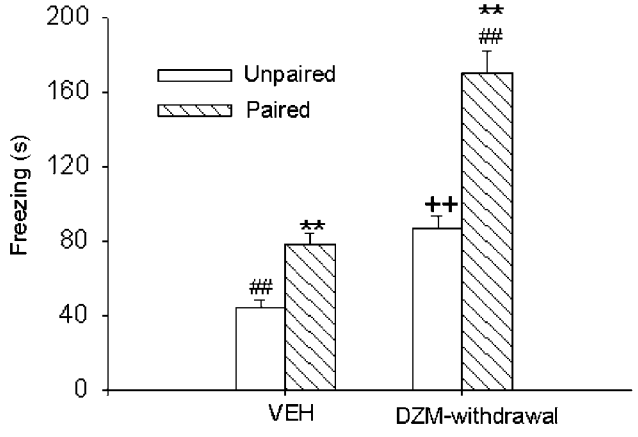

Figure 1 Effect of diazepam withdrawal on fear learning. Rats were pretreated daily for 21 days with VEH or diazepam (DZM, 2 mg/kg/i.p.). At 4 days after the last injection, all animals were submitted to a five-footshocks session and 24 h later evaluated in a novel, unpaired context or in the paired context. Values represent the mean (±SEM) time spent freezing during the 5-min test period ($n = 12$ per group). ** Significantly different from the respective unpaired group ($p < 0.01$). ++ Significantly different from VEH unpaired group ($p < 0.01$). ## Significantly different from all other groups ($p < 0.01$).

that fear conditioning is facilitated following early DZM withdrawal (ANOVA showed a significant effect of pretreatment × test condition interaction $F(1, 44) = 4.75$; $p = 0.0346$). Further, Newman–Keuls *post hoc* test revealed significant differences between paired and unpaired groups in both VEH and DZM withdrawal groups ($p < 0.01$). In addition, DZM withdrawal groups showed a significant increase in freezing as compared with VEH groups in both paired and unpaired conditions ($p < 0.01$). The analysis also showed that the freezing of DZM withdrawn rats in the paired context was higher than that from all remaining groups ($p < 0.01$). In addition, VEH animals in the unpaired context exhibited significantly less freezing as compared to the other groups. No other significant differences were detected. Analysis of time spent freezing during the pre- or postshock period revealed no significant differences between the experimental groups. The means ± SEM for the preshock period were: 9.8 ± 2.8 s for VEH and 12.0 ± 3.3 s for DZM withdrawal groups. The means ± SEM for the postshock period were: 107.4 ± 2.5 s for VEH and 106.6 ± 2.5 s for DZM withdrawal groups.

Electrophysiological Experiments

The evoked field response in vivo. In eight control animals with correctly placed stimulating and recording electrodes, single square pulses applied to the mPFC evoked a complex field response in the BLA that included a PS riding on a slower positive component usually followed by a negative slow phase (Figure 2a). Latency to peak for the PS was 20.3 ± 1.14 ms. In DZM withdrawn animals ($n = 8$) single stimuli applied to mPFC evoked a response with multiple spikes of decreasing amplitude (Figure 2a). Comparison of I/O curves for both groups yielded similar results ($F(1, 14) = 0.5$; $p = 0.5$) (Figure 2b). No differences were seen in the latency of the primary PS (*t* test; $p = 0.14$). These results suggested the possibility that inhibitory mechanisms that normally restrict repetitive firing in BLA (Wang *et al*, 2002, 2001) were affected in DZM withdrawn animals. The need to apply locally specific drugs for the analysis of the mechanism responsible of these changes and the possibility that the responses could be affected by the anesthetic used (Shirasaka and Wasterlain, 1995), induced us to continue this investigation in brain slices maintained *in vitro*.

The evoked field response in vitro. By placing the stimulating electrode in the EC, dorsal to the LA, we targeted incoming fibers of cortical origin (Fisk and Wyss, 2000; De Olmos *et al*, 1985). Needless to say, we cannot rule out the activation of other fibers in this stimulation site, including antidromic activation of the abundant amygdalocortical projections (De Olmos *et al*, 1985). In slices from control rats ($n = 28$) stimulation of the EC with single pulses evoked a complex field potential in the BLA comprising two fast negative-going components (fiber volley (FV) and PS) and occasionally a slow positive-going component (Figure 3d). FV had fast falling and rising phases with a peak latency of 3.9 ± 0.1 ms. PS was usually larger than FV and had a latency of 8.0 ± 0.2 ms; PS also showed rapid falling and rising phases. Increasing stimulus from threshold to supramaximal intensity evoked a parallel increase in the amplitude of the PS but in most cases did not modify the

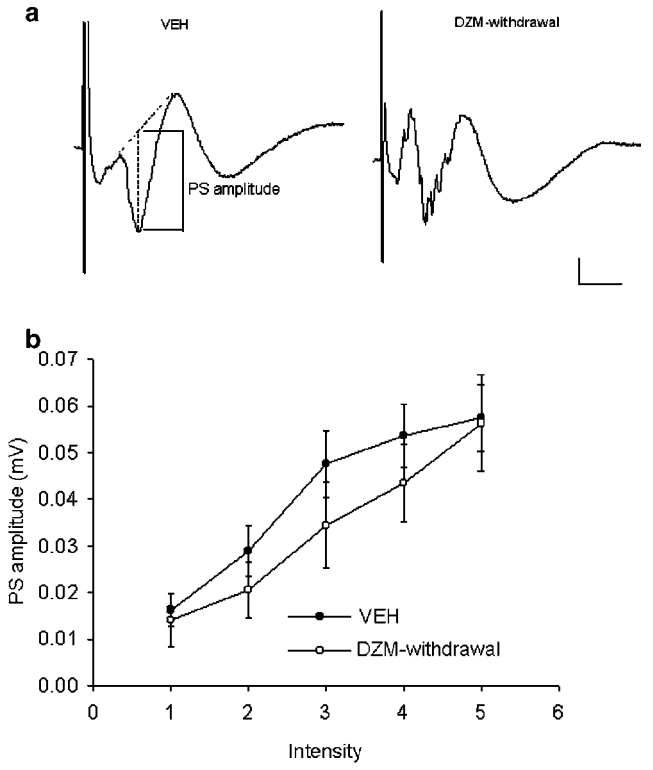

Figure 2 (a) Examples of extracellular field responses evoked in the BLA by stimulation of the medial prefrontal cortex recorded *in vivo* in VEH and DZM withdrawn rats. Note the repetitive PSs recorded in the latter. Dashed lines indicate the method to measure the amplitude of the PS; calibration bars: 0.01 mV and 20 ms. (b) Amplitude of PS as a function of stimulus intensity for VEH ($n=8$) and DZM withdrawn ($n=8$) rats.

overall response pattern; only in one out of 28 control cases, a third smaller negative spike (15 ms latency) appeared with the higher intensities used. In several cases, single unit responses could be recorded; in these cases, the evoked PS coincided with evoked firing of action potentials (Figure 3b). Keeping the stimulating electrode in the same EC site, the evoked response could be recorded from several places within the BLA nucleus, but disappeared when the recording electrode was placed outside the limits of the BLA (results not shown).

In order to identify the origin (pre- or postsynaptic) of the responses evoked in our preparation, the effects of $CdCl_2$, CNQX, and APV were studied. Perfusion of the slice with $CdCl_2$ 100 μM or CNQX 30 μM eliminated the PS and the slow component, whereas FV was unaffected (Figure 4); on the other hand, perfusion with APV 20 μM had little if any effect on the evoked responses. These results suggest that FV was either a fiber volley generated by the synchronous firing of stimulated incoming fibers from the EC or generated by the firing of antidromically stimulated cells, whereas PS was a postsynaptic response resulting from the stimulation of incoming fibers. Considering that blockade of Ca^{2+} currents and of glutamate AMPA-type receptors completely blocked the PS, plus the observation that single action potentials could be observed coinciding with the response, incline us to tentatively interpret it as a PS resulting from the summed action potentials of pyramidal neurons. The results obtained with paired pulse stimulation (see below) agree with this interpretation. In support of this possibility concurs the fact that the great majority (93%) of prefrontal-amygdala synapses identified in the BLA are located on dendritic spines of pyramidal projection cells and belong to the asymmetric (excitatory) type (Brinley-Reed *et al*, 1995). Moreover, EC stimulation has been shown to evoke monosynaptic excitatory post-synaptic potentials (EPSP) in BLA neurons (Chapman and Bellavance, 1992; Li *et al*, 1998; Mahanty and Sah, 1998; Chapman *et al*, 1990). Since interneurons represent a minority (<15%) of BLA neurons (McDonald, 1982; Millhouse and De Olmos, 1983), it is unlikely that their firing would have contributed significantly to the generation of the PS.

In slices obtained from rats after withdrawal of DZM ($n=23$) the same components (FV, PS, and slow component) were recorded. When the amplitude of the PS was plotted against stimulation intensity (I/O curves; Figure 3c), the response obtained in slices of DZM withdrawn rats was consistently smaller than in slices of control rats ($F(1,49)=5.66$; $p=0.02$). Moreover, in slices from DZM withdrawn rats the evoked response was characterized by the appearance of several (up to 5) additional fast spikes after PS (Figure 3d). These additional spikes appeared with variable latencies in individual records, but since the additional activity was partially synchronized, smaller secondary PSs of decreasing amplitude were observed in averaged records. The repetitive PSs observed in animals with DZM withdrawal were still observed even if stimulus intensity was reduced to near threshold levels. In other words, the repetitive response recorded from animals in DZM withdrawal was an all-or-nothing phenomenon with the same threshold as PS. PSs were counted in individual records of both groups and their numbers averaged across slices (one slice per rat) for the stimulation intensity that evoked the maximum response amplitude. The average number of PSs for DZM withdrawal group was significantly ($p<0.001$) larger than for the control group (2.83 ± 0.18 and 1.15 ± 0.06, respectively; Figure 3e).

Paired pulse stimulation. Paired pulse stimulation represents a tool for analysis of the excitation–inhibition cycle in neuronal networks (Marder and Buonomano, 2003), including the amygdala (Roberto *et al*, 2003; Wang *et al*, 2002; Rammes *et al*, 2000); short interstimulus interval-dependent paired pulse inhibition (PPI) is considered to depend on an IPSP that is $GABA_A$ mediated (Roberto *et al*, 2003; Marder and Buonomano, 2003; Delaney and Sah, 2001; Chapman and Bellavance, 1992; Braga *et al*, 2002; Adamec *et al*, 1981). Taking into account the results just described, we used PPI to evaluate the GABAergic inhibitory activity in BLA neurons, with the prospect of defining differences between control and withdrawn animals. Whereas in slices from control rats ($n=11$) a mean depression of $17.4\pm10\%$ was observed in the second response with interstimulus interval of 20 ms (Figure 5), no such inhibition was seen in slices from withdrawn animals. In fact in four out of eight slices from DZM withdrawn rats, facilitation was seen instead of inhibition with 20 ms of interpulse interval. MANOVA indicated a statistically significant effect associated to pretreatment ($F(1,3)=5.37$; $p=0.03$). *Post hoc* analysis indicated significant differences ($p<0.05$) with 20 and

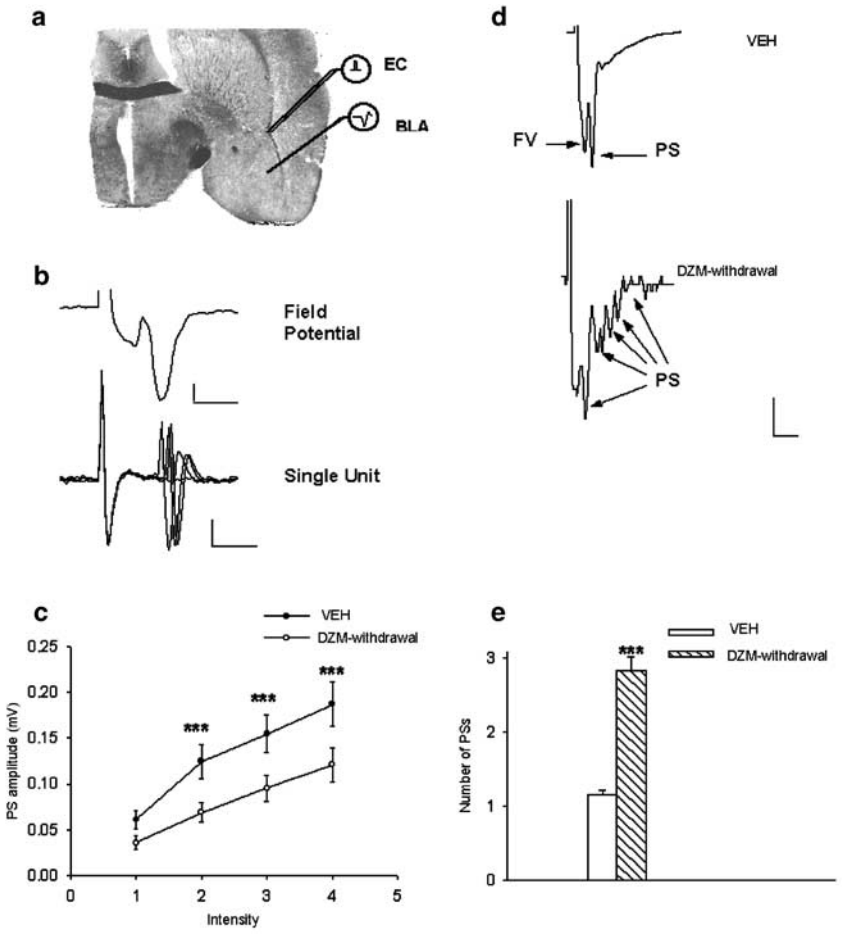

Figure 3 (a) Representative photomicrograph of a brain slice preparation indicating the BLA recording site and EC stimulation site. (b) Example of field potentials (upper record; average of 16 sweeps) and single unit response (lower record; note variable latency in three responses out of four sweeps) evoked in the BLA by stimulation of the EC in a slice from VEH treated rat. Calibration bars: 0.1 mV and 5 ms. (c) Amplitude of PS as a function of stimulus intensity for VEH and DZM withdrawn rats. *** Significantly different from VEH rats ($p<0.001$). (d) Example of field potentials evoked in BLA by stimulation of EC in slices from VEH (upper record) and DZM withdrawn rats (lower record). Calibration bars: 0.1 mV and 10 ms. (e) Mean (±SEM) number of PSs recorded in slices from VEH ($n=28$) and DZM withdrawn ($n=23$) rats. *** Significantly different from VEH ($p<0.001$).

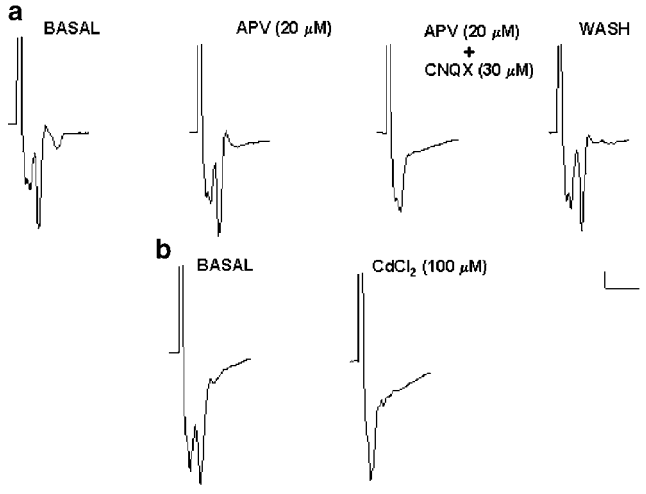

Figure 4 (a) Examples of extracellular responses evoked in the BLA by EC stimulation before (Basal), after perfusion with APV (20 μM) and APV (20 μM) plus CNQX (30 μM). (b) Examples of extracellular responses evoked in the BLA by EC stimulation before (Basal) and after perfusion with $CdCl_2$ (100 μM). Each trace is the average of 16 sweeps.

40 ms of interstimulus interval. This lack of PPI in withdrawn animals may be attributed to a decrease of the GABA-dependent IPSP that occurs simultaneously or following the EPSP evoked by EC stimulation (Wang *et al*, 2001; Rainnie *et al*, 1991b, a; Chapman and Bellavance, 1992). Interestingly, although the PS was not smaller in the response to the second stimulus, the secondary repetitive spikes were suppressed (Figure 5a). Neither facilitation nor inhibition was seen in FV at any of the interpulse intervals tested.

Effect of picrotoxin and diazepam. It has been demonstrated that activation of amygdala afferents in the EC (Chapman *et al*, 1990; Chapman and Bellavance, 1992) or in the LA (Rainnie *et al*, 1991b) elicit IPSPs that are superimposed or follow the EPSP of BLA neurons referred to above. These IPSPs are GABAergic and are elicited by direct or recurrent activation of local interneurons (Preleviç *et al*, 1976; Rainnie *et al*, 1991b); suppressing GABAergic transmission has been shown to result in increased excitability (Wang *et al*, 2002, 2001) and epileptiform activity (Chapman *et al*, 1990; Gean and Shinnick-Gallagher,

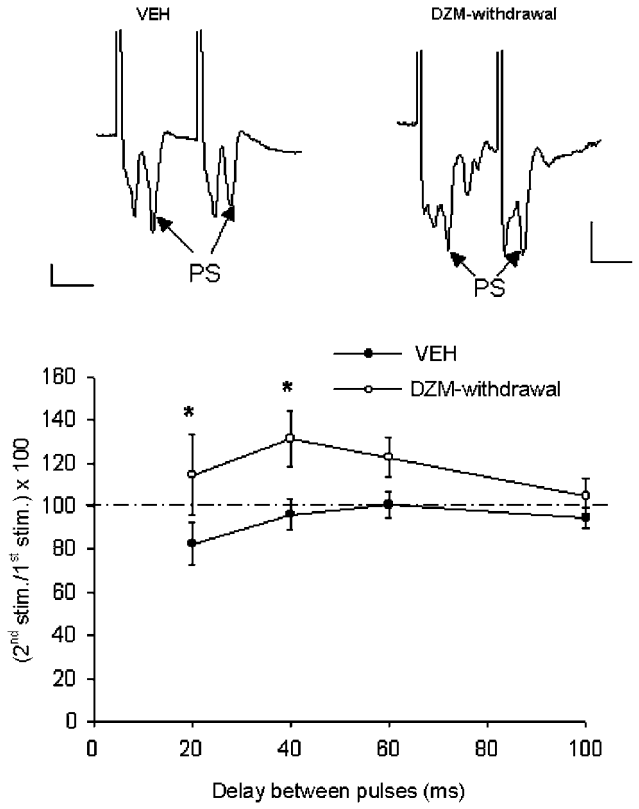

Figure 5 Paired pulse inhibition of the PS evoked in BLA by stimulation of the EC in slices from VEH ($n=11$) and DZM withdrawn ($n=8$) rats. Data are mean ($\pm$SEM) of the percent amplitude of the response to the second stimulus with respect to response to the first stimulus. Inset shows representative records obtained in slices from VEH (left) and DZM withdrawn (right) rats using an interstimulus interval of 20 ms. Each trace is the average of 16 sweeps. *Significantly different from basal ($p<0.05$). Calibration bars: 0.1 mV and 10 ms. Dotted line indicates 100%.

1987; Gean *et al*, 1989). The multiple PSs observed in our slices from DZM withdrawn rats suggested dysinhibition of BLA principal cells that may result from suppression of the GABAergic input. In order to study the consequences of GABAergic blockade in our preparation, slices obtained from VEH rats were perfused with PTX 50 μM. This treatment resulted in the appearance of multiple secondary PSs, similar to those recorded in slices from DZM withdrawn rats (Figure 6a). The effect of PTX was quantified by PS counting and was statistically highly significant (basal: 1.2 ± 0.13 PSs; after PTX: 4.0 ± 0.4 PSs; $p<0.001$; $n=6$). On the other hand, perfusion with PTX of slices from DZM withdrawn rats had no evident effect on the number of PSs (basal: 3.1 ± 0.2 PSs; after PTX: 3.7 ± 0.3 PSs; $n=8$). Perfusion with PTX had no effect on the amplitude of the PS on the DZM withdrawal group (basal: 0.06 ± 0.01 mV; after PTX: 0.07 ± 0.01 mV; Wilcoxon's matched pairs test; $p=0.12$) nor in the VEH group (basal: 0.2 ± 0.07 mV; after PTX: 0.2 ± 0.09 mV; $p=0.22$). Treatment with PTX had no effect on the amplitude of the FV on the DZM withdrawal group (basal: 0.08 ± 0.01 mV; after PTX: 0.08 ± 0.01 mV; $p=0.86$) nor in the VEH group (basal: 0.08 ± 0.03 mV; after PTX: 0.08 ± 0.03 mV; $p=0.22$).

To test the possibility that potentiation of GABAergic input may normalize the response in DZM withdrawn rats, slices were perfused with DZM 2 μM. After 30 min of perfusion with the positive GABA modulator, the multiple responses observed during the control period had decreased or disappeared and in most cases a single PS characteristic of control slices was evoked by EC stimulation (Figure 6b). Counting PSs objectively demonstrated the effect of DZM perfusion in slices from DZM withdrawn rats (basal: 3.3 ± 0.1 PSs; after DZM: 1.6 ± 0.2 PSs; $p<0.001$; $n=7$). Perfusion of slices from control rats with DZM had no effect on the number of PSs (basal: 1.3 ± 0.1 PSs; after DZM: 1.2 ± 0.1 PSs; $n=11$). Perfusion with DZM had no effect on the amplitude of the PS on the DZM withdrawal group (basal: 0.07 ± 0.02 mV; after DZM: 0.07 ± 0.02 mV; Wilcoxon's matched pairs test; $p=0.5$) nor in the VEH group (basal: 0.14 ± 0.04 mV; after DZM: 0.13 ± 0.04 mV; $p=0.92$). Perfusion with DZM had no effect on the amplitude of the FV on the DZM withdrawal group (basal: 0.08 ± 0.03 mV; after DZM: 0.09 ± 0.03 mV; $p=0.68$) nor in the VEH group (basal: 0.09 ± 0.03 mV; after DZM: 0.09 ± 0.03 mV; $p=0.65$).

Induction of LTP. In view of the results just described and considering that increased neuronal excitability facilitates LTP induction in BLA (Krezel *et al*, 2001; Rammes *et al*, 2000), we were prompted to investigate whether in DZM withdrawn animals induction of LTP could be different from control rats. Pilot experiments confirmed previous reports showing that a single train of HFS in the EC (Li *et al*, 1998; Chen *et al*, 2003) or LA (Rammes *et al*, 2000) was not sufficient to induce LTP in the BLA of normal rats. Therefore, we tested the effect of one train (1 s at 100 Hz) in a different set of control and DZM withdrawn rats. In slices from control rats a modest and nonsignificant increase ($39.45\pm22.14\%$; $p=0.99$; $n=11$; Figure 7) was observed in the amplitude of the PS 40 min after HFS. In slices from DZM withdrawn rats ($n=9$), already 10 min after stimulation the amplitude of the PS had almost doubled and continued to increase up to 250% at 40 min (Figure 7). The evoked response remained potentiated for the duration of the experiment, 2 h after stimulation. The overall ANOVA showed a highly significant effect of treatment ($F(1,20)=26.9$; $p=0.00004$). *Post hoc* comparisons showed that in slices from DZM withdrawn rats the increase in PS amplitude was statistically highly significant ($p<0.001$) at all times recorded after HFS. Apart from the increase in PS amplitude, the amplitude of the secondary additional repetitive responses was also potentiated in withdrawn rats (Figure 7a); the amplitude and number of secondary spikes were not quantified. Induction of LTP had no effect on the amplitude of the FV on the DZM withdrawal group (basal: 0.05 ± 0.01 mV; after HFS: 0.06 ± 0.01 mV; Wilcoxon's matched pairs test; $p=0.24$) nor in the VEH group (basal: 0.08 ± 0.01 mV; after HFS: 0.08 ± 0.01 mV; $p=0.85$). These results suggest that increased excitability of pyramidal neurons due to suppression of GABAergic inhibition (dysinhibiton) facilitates induction of LTP in DZM withdrawn animals.

DISCUSSION

The results described above demonstrate that after withdrawal from chronic DZM, rats that display enhanced fear

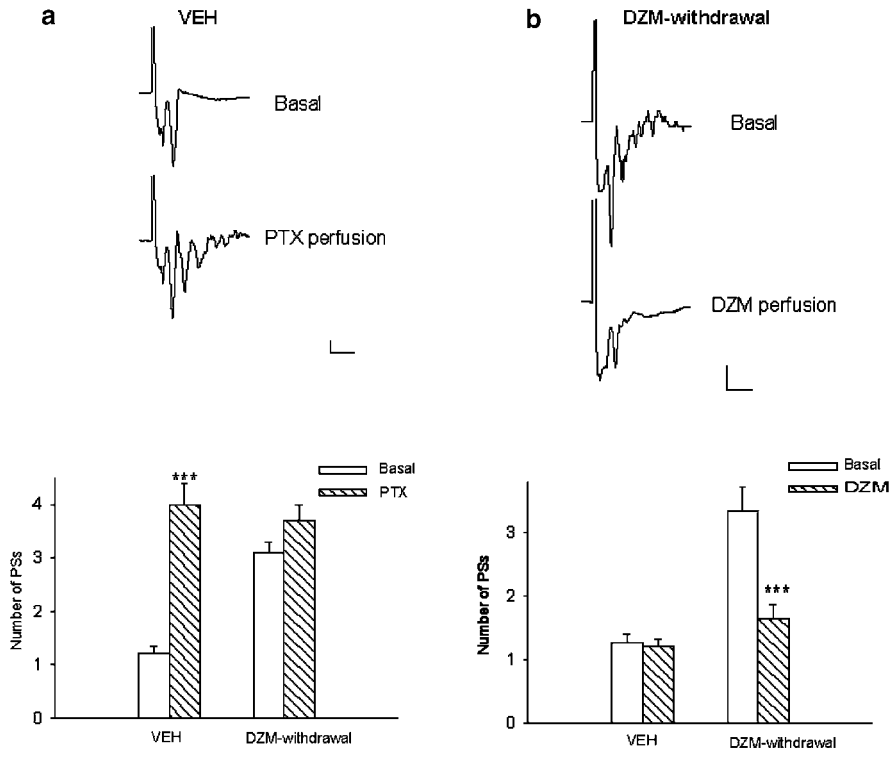

Figure 6 (a) Effect of perfusion with PTX (50 μM) on the number of PSs evoked in the BLA by EC stimulation. The values are mean (±SEM), for six VEH and eight DZM withdrawn rats. *** Significantly different from basal ($p<0.001$). The inset shows representative traces from VEH rats before (upper record) and after 15 min perfusion with PTX (lower record). (b) Effect of perfusion with DZM (2 μM) on the number of PSs evoked by EC stimulation in the BLA. The values are mean (±SEM), for 11 VEH and seven DZM withdrawn rats. *** Significantly different from basal ($p<0.001$). The inset shows representative traces from DZM withdrawn rats before (upper record) and after 30 min perfusion with DZM. Calibration bars: 0.1 mV and 10 ms.

learning also show increased neuronal excitability and facilitated LTP in BLA. In support of numerous reports (see Fendt and Fanselow, 1999; Davis and Whalen, 2001; Maren, 2001), the present behavioral findings show that both groups of animals – control and DZM withdrawal – exposed to the context previously paired with footshock, exhibited significant more freezing in comparison to animals exposed to the unpaired context, indicating a reliable conditioned freezing in the associated chamber. Our results show, in addition, that rats exposed to DZM discontinuation freeze significantly more than control rats in response to the associated contextual cue. These results indicate that DZM discontinuation does not affect the initial formation of fear memory but rather influences the formation of long-term memory, since a similar freezing response was observed in control and DZM withdrawn animals during the postshock period. However, a negative effect of DZM withdrawal on the extinction phase during the test, that is, delayed extinction of freezing, could also contribute to the enhancement of fear conditioning observed in withdrawn animals. Further experiments are necessary to elucidate this possibility. In addition, since withdrawn rats also showed more freezing than control rats when exposed to the unpaired context, an influence on the subsequent nonassociative response or a sensitized reactivity to the US occurs. It is clear that DZM withdrawn animals displayed an increased fearful response expressed both as associative freezing and as nonassociative freezing in response to a novel environment following the exposure to an uncontrollable footshock experience. It could be argued that differences in associative and nonassociative freezing between control and DZM withdrawn rats may be caused by an influence of DZM withdrawal on pain threshold. However, a similar freezing response was observed between DZM withdrawn rats and control animals during the postshock observation period. Moreover, previous findings showed an altered behavior in response to a stressful situation without a painful component (forced swimming experience) in animals exposed to DZM withdrawal (Martijena *et al*, 1996, 1997). In addition, preliminary evidence from this laboratory showed an enhanced anxiogenic response in the elevated plus maze following exposure to this nonpainful stressful event in DZM withdrawn animals as compared to VEH treated rats. Taken all together, these findings support the view that differences in behavioral freezing between control and DZM withdrawn rats observed in the present study are not due to an alteration of the nociceptive threshold.

Besides the fact that subnuclei of the amygdala may be differently involved in the mediation of associative and nonassociative fear responses (see Fendt *et al*, 2003), a large body of evidence agrees with the idea that the BLA is involved in emotional pavlovian learning, neural plasticity, and storage of emotional memory. In turn, this learned information critically influences the coordination of emotional responses mediated by a number of brain structures (Maren, 2001). Therefore, the fact that DZM withdrawal induced a clear facilitation of pavlovian fear learning, as

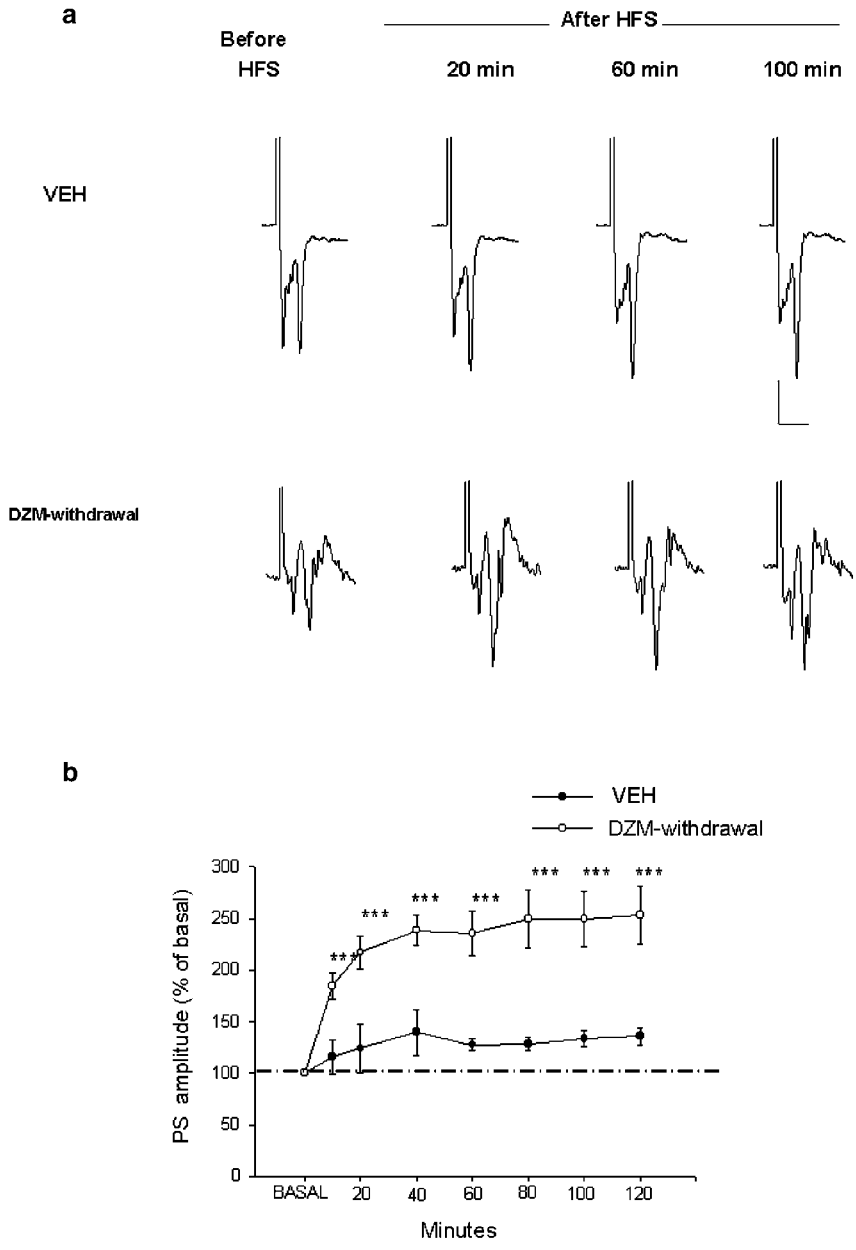

Figure 7 (a) Examples of extracellular responses evoked by EC stimulation in the BLA before and 20, 60, and 100 min after high frequency stimulation (HFS) in slices from VEH (upper row) and DZM withdrawn (lower row) rats. (b) Effect of HFS applied in the EC on the amplitude of the PS recorded in the BLA; PS amplitude is expressed as percent of control record (basal) obtained in the same slice before HFS. The values are mean (±SEM), for slices obtained from 11 VEH and nine DZM withdrawn rats. *** Significantly different from basal records ($p < 0.001$). Dotted line indicates 100%.

reflected by our conditioning behavioral data, suggests that previous DZM withdrawal could affect synaptic neurotransmission in the BLA.

Whereas in control rats anethetized with urethane single pulse stimulation of the mPFC evoked a single PS in the BLA, in DZM withdrawn rats the same stimulation evoked multiple PSs from BLA neurons. Coincidently, in slices from control rats EC stimulation evoked a single PS, whereas in slices from DZM withdrawn rats multiple PSs were evoked. Cortical afferents to the BLA can control the firing of pyramidal projection neurons by several mechanisms that depend on the feedforward and recurrent recruitment of GABAergic interneurons (Rosenkranz and Grace, 1999, 2002). Data obtained *in vivo* have demonstrated a powerful control through GABAergic inhibition over the activity of projecting principal cells (Lang and Pare, 1997, 1998) that renders a special role to the GABAergic interneurons in the control of excitation in this region. The repetitive responses observed could in theory be explained by at least two mechanisms: potentiated synaptic transmission or decreased inhibition of postsynaptic neurons. Since in the I/O curves no evidence was found of potentiated synaptic

responses in DZM withdrawn rats, the repetitive response observed in these rats suggests dysinhibition of pyramidal neurons originated in depression of recurrent GABAergic input from BLA interneurons. The I/O curves obtained *in vitro* actually showed depression of the response amplitude in DZM withdrawn rats, whereas the experiments made *in vivo* gave marginal results, since VEH injected animals have higher values than animals injected with DZM but the differences were not significant, probably because the number of animals is small and there was greater individual variation. At first sight, depression of the response amplitude appears contradictory with dysinhibition resulting from depressed GABAergic transmission, but it should be kept in mind that a reduction of *recurrent* inhibition would not necessarily increase synaptic response amplitude. A reduction of recurrent inhibition would, however, facilitate repetitive firing and decrease inhibition in the PPI paradigm, a result we observed in DZM withdrawn rats. Moreover, alterations in glutamatergic transmission resulting from DZM withdrawal could help explain these results (for a review see Allison and Pratt, 2003). Following DZM withdrawal, significant reductions in the expression of AMPA receptor subunit mRNA in the hippocampus and lateral nucleus of the amygdala have been reported (Allison and Pratt, 2003). Alternatively, feedforward inhibition, which would affect results in the I/O curves, may not necessarily be depressed by DZM withdrawal as appears to be the case with recurrent inhibition. It would be necessary to explore these possibilities in future studies.

The fact that perfusion of slices from DZM withdrawn rats with a positive GABA modulator normalized the evoked response, agrees with the expected effect of DZM, namely increased amplitude and increased duration of the effect of locally released GABA (Mody *et al*, 1994), and further supports the proposition that the observed hyperexcitability can be attributed to decreased GABAergic inhibition of principal neurons. Coincidently, blockade of $GABA_A$ receptors induced the same repetitive activity in slices from normal rats (our results) or after tetanization (Rammes *et al*, 2000). The fact that in slices from DZM withdrawn rats facilitation was observed instead of inhibition in the PPI paradigm point to the same interpretation. All together, these results indicate that by altering the equilibrium between excitation and inhibition, withdrawal from DZM administration results in dysinhibition of BLA projection neurons, thus favoring multiple firing in response to cortical afferent activity. It is appropriate to recall that recurrent inhibition was depressed after BDZ withdrawal in the spinal cord and the hippocampus; no similar data have previously been reported for the amygdala (for a review see Wilson, 1996). In a model of 'GABA withdrawal' induced by the suspension of treatment with GABA or GABAergic drugs both *in vitro* and *in vivo*, work from Brailowsky's laboratory (Brailowsky, 1991; Davies *et al*, 1988) has reported similar results for the hippocampus and cerebral cortex. Owing to methodological considerations, results are not strictly comparable; however, in both cases hyperexcitability is made evident by repetitive spikes evoked by a single stimulus. Recently, Casasola *et al* (2001) have shown that this hyperexcitability can be explained by the decreased number of $GABA_A$ receptors observed in the CA1 region during 'GABA withdrawal'. Notably, also after alcohol withdrawal, signs of neural dysinhibition have been described in the hippocampus (Geisler *et al*, 1978) and attributed to alterations in the sensitivity of $GABA_A$ receptors (Kang *et al*, 1998). In contrast to the BDZ withdrawal syndrome, after alcohol withdrawal there is increased sensitivity to excitatory input in the hippocampus, probably caused by an adaptive upregulation of NMDA receptor function (Samson and Harris, 1992).

The behavioural consequences of BDZ withdrawal (increased aversive learning) as shown in this report are expected to depend on changes of neurophysiological mechanisms sustaining memory and learning in the amygdala (Tsvetkov *et al*, 2002; Rogan *et al*, 1997; McKernan and Shinnick-Gallagher, 1997; Maren, 1999). The closest experimental approximation to such mechanisms is LTP, so it can be expected that induction of LTP will be facilitated in animals under DZM withdrawal. Our results support this expectation, since LTP was generated by a stimulation paradigm that was insufficient in control animals. The mechanism for the facilitation of LTP induction appears straightforward: if GABAergic inhibition of responses to cortical input is depressed (dysinhibiton), the probability of coincidence between postsynaptic depolarisation and presynaptic activation should increase, which in terms of spike timing explains sustained potentiation of the synaptic response (Shumyatsky *et al*, 2002). Indeed, the facilitatory effect of GABAergic suppression on LTP induction has been previously demonstrated (Watanabe *et al*, 1995; Rammes *et al*, 2000). In summary, dysinhibition produced as a consequence of DZM withdrawal would result in greater synaptic plasticity leading to increased aversive learning. Work in progress shows that after immobilization stress, which also generates a state of increased anxiety, the same parameters as used here evoke multiple PSs and induce LTP (Rodriguez *et al*, 2003). The behavioral response to stress was mimicked by bicuculline injection in the BLA, whereas pretreatment with midazolam eliminated the effects of stress or bicuculline injection. These findings suggest that the results obtained in rats after withdrawal of BDZ may be characteristic of increased emotional states generated by an environmental challenge, such as stress or withdrawal.

The degree of excitability of pyramidal projecting neurons from the BLA is functionally relevant to transfer emotional information to a series of target areas that will mediate the appropriate signs and symptoms of fear and anxiety in response to a particular environmental stimulation (Maren, 2001). Hence, the modulatory role on excitability in the BLA mediated by $GABA_A$ receptors could be critically involved in the regulation of the emotional response to a challenge stimulus. Consonant with such view, a number of studies have shown a high density of BDZ sites in the BLA (Niehoff and Kuhar, 1983) and a clear anticonflict effect after the local infusion of BDZ in the BLA (see review by Davis, 1997). Moreover, the pharmacological stimulation of $GABA_A$ sites or the stimulation of GABAergic neurotransmission in the BLA induced a robust decrease of fear learning (Wilensky *et al*, 2000). In fact, inactivation of BLA neurons with a $GABA_A$ receptor agonist reduces both the acquisition and expression of fear conditioning (Helmstetter and Bellgowan, 1994; Muller *et al*, 1997; Wilensky *et al*, 1999). Although abundant

evidence has shown that $GABA_A$ sites located in projecting pyramidal neurons are critical for an inhibitory control of the BLA excitability (Rainnie *et al*, 1991b; Sugita *et al*, 1993; Washburn and Moises, 1992), McDonald and Mascagni (1996) reported an intense $GABA_A$ receptor-like immunoreactivity in a subpopulation of nonpyramidal neurons which could play an important dysinhibitory role. In order to reconcile this apparent opposite functional roles of $GABA_A$ receptors in BLA, McDonald and Mascagni (1996) proposed that part of the intense $GABA_A$ receptor-like immunoreactivity in BLA could represent $GABA_A$ sites associated effectively with distal dendrites of pyramidal neurons.

It is widely known that the potentiation of $GABA_A$ receptor-mediated inhibition underlies the therapeutic efficacy of BDZ agents. Although there is no definitive consensus concerning the influence of chronic BDZ administration on $GABA_A$ receptor functioning (Pratt *et al*, 1998; Bateson, 2002), numerous findings evidenced that prolonged exposure to positive modulators of BDZ sites such as DZM can result in altered $GABA_A$/BDZ receptors, which may lead to reduced GABAergic neurotransmission. Indeed, a number of authors have proposed that this reduction is due either to altered coupling of the allosteric linkage between GABA and BDZ sites or alteration of receptor cycling or to modifications of diverse subunits mRNA levels (see review by Bateson, 2002). Whatever the molecular mechanism involved, it is reasonable to propose that prolonged DZM administration attenuates the inhibition mediated by $GABA_A$ sites in BLA. It is widely accepted that withdrawal signs are the outcome of neuroadaptive mechanisms developed during repeated exposure to drugs of abuse and opposite to the effects observed following acute exposure (Markou *et al*, 1998; Kreek and Koob, 1998). Therefore, following the abrupt removal of the BDZ ligand, the attenuated inhibition resulting from 'altered' GABAergic neurotransmission could release projecting neurons in the BLA, explaining the observed emotional disturbances in DZM withdrawn animals. In support of an enhanced excitability of BLA neurons following withdrawal, Pratt *et al* (1998) demonstrated a clear increase in glucose utilization in brain regions of the emotion circuit, including the BLA, following DZM discontinuation. In summary, increased fear learning, neuronal hyperexcitability and facilitated LTP could be partially explained by depressed GABAergic recurrent inhibition in BLA resulting from abrupt discontinuation of chronic DZM administration.

A number of authors have proposed that negative affective states including negative emotions such as irritability, anxiety, and depressive symptoms are common to withdrawal from chronic use of most drugs of abuse, including hypnotic-sedative agents (see Kreek and Koob, 1998). In addition, these emotional states could be major motivational factors contributing to the maintenance of addiction (Koob and LeMoal, 2001). Furthermore, considering the well-established role of stress in reinstating addictive behavior and relapse following abstinence (Sinha, 2001; Shaham *et al*, 2000), an emotional hyperreactivity and a facilitated fearful response to stress as observed in DZM withdrawn animals might be critical factors in precipitating relapse after discontinuation from these drugs.

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