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Alterations in GABAergic function following forced swimming stress

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Abstract

Forced swimming induces alterations in the GABA brain concentration and could change the sensitivity of the GABA/benzodiazepine receptor–chloride ionophore complex to benzodiazepines. This change in sensitivity could be explained by the allopregnanolone release that takes place during stress. The current study was carried out to determine whether forced swimming is able to modify the anti-anxiety effect of diazepam and to explore the possible relation of this change to allopregnanolone, the GABA concentration or/and the GABA/benzodiazepine receptor density.

Unstressed and stressed mice, injected with the vehicle or diazepam, were evaluated in the exploratory behavior test. Diazepam induced clear anxiolytic actions at all doses in unstressed animals, but such an effect was not observed in stressed animals. The injection of allopregnanolone 24 h before the anxiety test blocked the effect of this benzodiazepine. Forced swimming decreased GABA concentrations in the hippocampus and the thalamus–hypothalamus region, besides decreasing the [^{3}H]flunitrazepam labeling in both the hypothalamus and amygdala.

These results show that forced swimming abolishes the anti-anxiety effect of diazepam.

Keywords: Stress; Forced swimming; GABA; Mice; Anxiety; Diazepam

1. Introduction

The effects of environmental stress on the central gamma-amino-butyric acid (GABA) mediated neurotransmission have been extensively studied in animals by using biochemical and behavioral techniques. Acute stressors have been reported to either increase (Saulskaya and Marsden, 1995; Harvey et al., 2002) or decrease (Sherman and Gebhart, 1974; Otero Losada, 1988) brain GABA levels. Other studies have reported an augmented function of the brain GABA system following heavy swim stress (Soubrie et al., 1980; Skerritt et al., 1981; Akinci and Johnston, 1993, 1997). Several independent laboratories have also demonstrated that stress alters the functional properties of GABA/benzodiazepine receptor–chloride ionophore complex and/or the number of such sites in the nervous system (Schwartz et al., 1987; Otero Losada, 1988; Drugan et al., 1989; Rago et al., 1989; Montpied et al., 1993). In this sense, stressful handling of rats decrease the convulsive activity of several GABA/benzodiazepine receptor ligands such as bicuculline (Drugan et al., 1985), picrotoxin and pentylenetetrazole (Soubrie et al., 1980; Abel and Berman, 1993; Pericic et al., 2001). Therefore, it is unquestionable that the GABAergic activity is modified by severe stress, and that such change can be related to alterations in the pharmacological profile of drugs interacting with the GABA/benzodiazepine complex.

Forced swimming is one of the main stressful factors employed to understand changes at GABA/benzodiazepine receptor (Deutsch et al., 1994; Pokk et al., 1996; Marin et al., 1996; Pericic et al., 2000, 2001; Avital et al., 2001). For instance, this stressor is able to attenuate the anti-seizure efficacy of flurazepam (Deutsch et al., 1990). Based on the observation that acute stress influences the GABA/benzodiazepine receptor functionality, the main purpose of this

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investigation was to test the capability of forced swimming to modify the behavioral effect of diazepam. To this end, stressed mice injected with several doses of this agent were observed in the exploratory behavior test and the results were compared with those of unstressed animals.

Evidence shows that the progesterone $GABA_A$-modulatory metabolite 5α-pregnan-3α-ol-20-one (allopregnanolone) is released and rapidly metabolized after an acute stress session such as forced swimming (Purdy et al., 1991). It has been confirmed that this release occurs in both rats (Barbaccia et al., 1996, 2001) and mice (Mele et al., 2004) when using other stressful stimuli other than swimming. Accordingly, it has been reported that acute stress increases the peripheral-type benzodiazepine receptor density, and such receptor plays a major role in steroidogenesis (Weizman and Gavish, 1993; Cavallaro et al., 1992). Some authors have found that high doses of allopregnanolone (Gulinello et al., 2001) as well as its abrupt withdrawal (Smith et al., 1998) increase the synthesis of α_4 mRNA (one of the subunits that constitute the $GABA_A$ receptor). Interestingly, several studies have demonstrated that the presence of the α_4 subunit in the $GABA_A$ receptor decreases its sensitivity to benzodiazepines (Wisden et al., 1991; Knoflach et al., 1996; Wafford et al., 1996). From this evidence, it is likely that forced swimming could produce isomeric changes in the $GABA_A$ receptor complex that, at the same time, could alter the pharmacological response to benzodiazepines. Trying to mimic the abrupt release of allopregnanolone that supposedly takes place after stressing, we administered a high dose of this hormone to unstressed mice and evaluated their behavior 24 h later in the exploratory behavior test.

As mentioned, acute stress seems to modify the GABA levels and the functionality of the GABAergic transmission. In order to explore this putative consequence, both GABA concentration and GABA/benzodiazepine receptor density were analyzed in several brain areas of mice previously stressed by forced swimming. Hence, mice were sacrificed and their brain analyzed by means of autoradiographic techniques and high performance liquid chromatography (HPLC). Since to stress mice showed the lowest anxiety levels 24 h after swimming in the exploratory behavior test (Briones-Aranda et al., 2002), all experiments were carried out at this time.

The brain regions studied were chosen because they have been implicated in both anxiety and stress regulation (Shibata et al., 1989; Bowers et al., 1998; Koyama et al., 1999; Jardim and Guimaraes, 2001; Herman et al., 2002; Cook, 2004).

2. Materials and methods

2.1. Animals

Swiss Webster adult male mice (25–30 g) were used. Mice were bred in our laboratory, housed in groups of 10 animals each in plastic cages (44×21×21 cm) and submitted to 12:12 h inverted light cycle (10:00 off, 22:00 on). Food and water were available ad libitum at all times. All procedures were conducted in accordance with the Mexican Official Norm for Animal Care and Handling (NOM-062-ZOO-1999) as approved by the Institutional Ethics Committee of CINVESTAV-IPN, México.

2.2. Forced swimming stress

This process was carried out by using a modified version of the animal model proposed by Porsolt et al., (1977a,b) as a validated tool to screen agents with antidepressive activity. This paradigm consists of an escape-proof Plexiglas cylinder (25 cm high, 10 cm in diameter) containing 15 cm of water at 25 °C where each mouse was forced to swim. After the stressing session (15 min), the animal was dried, warmed up, and returned to its home cage. In all cases the water in the tank was changed every two sessions.

2.3. Anxiety paradigm

The avoidance exploratory behavior test is a broadly used procedure to study experimental anxiety and to screen drugs with potential anxiolytic activity. This model consists of an acrylic cage (44×21×21 cm) divided into a small, darkened compartment (1/3 of total size) and a large and highly illuminated (560 lx light intensity) compartment (2/3). A little opening (13×15 cm) separated the dark area from the bright one. In this test each mouse was introduced (only once) into the bright area and the number of transitions throughout the opening was registered for 10 min. Thus, an increase in the number of transitions was interpreted as an anxiolytic effect (Crawley and Goodwin, 1980). After each session the test cage was carefully cleaned with a moist cloth.

2.4. Activity test

For controlling results in the anxiety test due to alterations in motor activity, a spontaneous ambulatory behavior test was conducted after the anxiety test. Hence, the animal was placed into an acrylic cage (60×40×40 cm) that had a checkerboard pattern (20×20 cm) on the floor, and the total number of squares crossed by the mouse was manually registered for 10 min.

2.5. Drugs

The drugs used in this study were: diazepam and allopregnanolone (Sigma, St. Louis, Mo., U.S.A.). All drugs were injected i.p. at a total volume of 4 ml/kg. Diazepam was dissolved in propylene glycol 40%. Allopregnanolone was dissolved in beta-cyclodextrin (5%). Doses and latencies were chosen considering previous

studies (Lopez-Rubalcava et al., 1992; Fernandez-Guasti and Picazo, 1995; Gulinello et al., 2001).

2.6. High performance liquid chromatography

Independent groups of intact and stressed (24 h before) mice were used. Hence, the brains were rapidly removed and the following areas were dissected on an ice-cold plate: hippocampus, thalamus–hypothalamus and frontal cortex (Carlsson and Lindqvist, 1973; Oka et al., 1982). GABA tissue content was analyzed with an HPLC fluorometric detection procedure according to the method described by Kendrick et al. (1988). Briefly, homogenate was derivatized with *ortho*-phthaldehyde prior to injection into the HPLC apparatus. The *ortho*-phthaldehyde derivatizing agent was prepared by adding 15 mg of *ortho*-phthaldehyde, 300 μl of methanol, 2.8 ml of potassium tetraborate and 25 μl of mercaptoethanol. A 6 μl aliquot of *ortho*-phthaldehyde reagent was reacted with 20 μl of the homogenate for 2 min. The *ortho*-phthaldehyde-amino acid adducts were resolved on a reversed-phase 3.9×150 mm column (Nova-pack, particle size 4 μm, C18) with eluent A (sodium acetate 39.74 mM and methanol 10%, pH 5.7) and eluent B (sodium acetate 7.95 mM and methanol 80%, pH 6.7). The flow rate was 0.5 ml/min with a gradient profile as follows: 23–45% in 3 min, 45–74% in 6 min, 74–97% in 3 min, 97–23% in 8 min; complete analysis required 20 min. A fluorescence detector (Waters 474, 360 nm λ excitation and 450 λ emission) was used. The limit of detection was 2 nMol.

2.7. Auto-radiography for GABA/benzodiazepine receptors

The brains of unstressed and stressed (24 h before) mice were quickly removed, frozen, and stored at −70 °C. Brains were cut in coronal sections (20-μm thick) in a cryostat, mounted on gelatin-coated slides and again stored at −70 °C until processed.

Tris–citrate (50 mM; pH 7.4) and Tris HCl (170 mM; pH 7.4) buffers were used for GABA/benzodiazepine receptors auto-radiography incubations. In accordance with Rocha et al. (1994), the brain sections were pre-washed for 30 min at 25 °C. The sections were subsequently incubated for 45 min at 4 °C in a solution containing 2 nM [^{3}H]flunitrazepam (82.5 Ci/mM) and buffers, either in the presence or absence of 1 μM of chlordiazepoxide. The binding obtained in the presence of chlordiazepoxide was considered to be non-specific. Finally, incubation was stopped with two consecutive washes (1 min each) in the buffer and in a distilled water rinse (2 s) at 4 °C. The sections were then quickly dried under a gentle stream of cold air.

The slides were arrayed in X-ray cassettes together with tritium standards (Amersham), and opposed to tritium-sensitive film (Amersham Hyperfilm) for 3 weeks (benzodiazepine binding) at room temperature. [^{3}H]flunitrazepam activity was analyzed in the following structures: frontal cortex, hippocampus, dentate gyrus, thalamus, hypothalamus, and amygdala. The films were developed using standard Kodak GBX and fixer at room temperature.

Different brain regions were identified according to Paxinos and Franklin (2002), and the optical density was determined by means of a video-computer enhancement program (JAVA Jandel Video Analysis Software). For each area, 10 optical density readings were taken from at least six sections, and were averaged. The optical density readings of the standards were used to determine tissue radioactivity values for the accompanying tissue sections and to convert them to fmol/mg tissue.

2.8. Procedure

2.8.1. Experiment 1

To evaluate the influence of stress on the anxiolytic properties of diazepam, both stressed and unstressed mice received various doses of diazepam (0.0, 0.125, 0.25, 0.5 and 1.0 mg/kg). Their behavior was registered in the anxiety test 24 h after stressing. Each dose was assayed in ten animals. The spontaneous ambulatory behavior test was conducted immediately after the exploratory behavior test. All data were analyzed by using a two-way analysis of variance, taking into account the stress condition as factor "A", while drug treatment was considered as factor "B". Post hoc comparisons were done with the Student Newman–Keuls method.

2.8.2. Experiment 2

Forty intact mice were injected 24 h previously with the neurosteroid allopregnanolone (two groups of 10 mice with 10 mg/kg, two groups of 10 mice with 20 mg/kg). One group of 10 mice injected with 10 mg/kg of allopregnanolone was given diazepam (0.5 mg/kg −30 min) and the other group was given the vehicle (propylene glycol 40% −30 min) before the anxiety test. In the same way, one group of ten mice injected with 20 mg/kg of allopregnanolone was given diazepam (0.5 mg/kg −30 min) and the other group was given the vehicle before the anxiety test. Data from this experiment were compared with those from a control group (n=10) receiving beta-cyclodextrin (−24 h) and propylene glycol (−30 min) before being tested in the exploratory behavior test. As in experiment 1, a spontaneous ambulatory behavior test was conducted after this evaluation. Data from this experiment was analyzed by means of one way analysis of variance.

2.8.3. Experiment 3

A group of six mice was exposed to forced swimming for 15 min and sacrificed by decapitation 24 h later; another unstressed group (n=6) was used as control. Specific brain regions were dissected in order to measure the GABA concentration. Results from this experiment were analyzed by means of the Student t test.

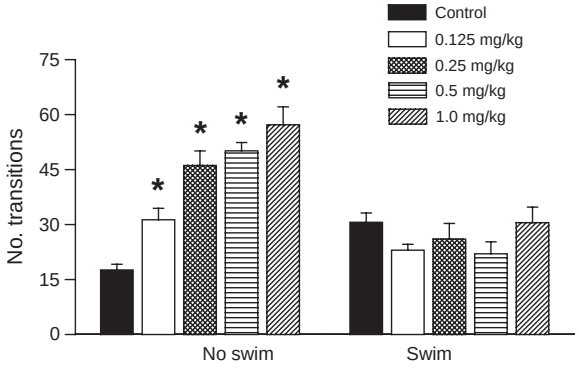

Fig. 1. Effect of diazepam on the number of transitions in the exploratory behavior test of stressed and unstressed mice. Each column represents the mean±S.E. of 10 animals. Student Newman–Keuls method, *p<0.05 vs. the corresponding control.

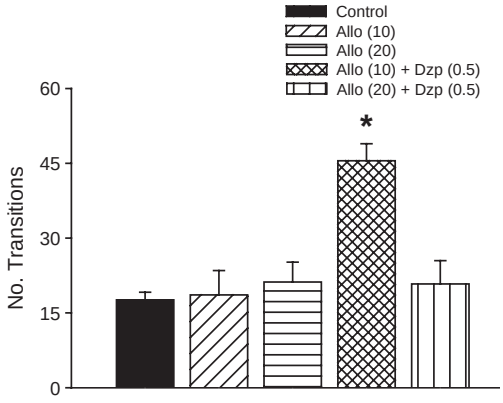

Fig. 2. Effect of diazepam on the number of transitions in the exploratory behavior test of mice observed 24 h after the allopregnanolone injection in comparison with a group treated with the vehicles. The number in parenthesis represents the dose used in mg/kg. Allo=allopregnanolone; Dzp=diazepam. Data are expressed as the mean±S.E. *p<0.05 vs. the control group.

2.8.4. Experiment 4

A group of seven mice was exposed to forced swimming for 15 min and sacrificed by decapitation 24 h later; another without stressing group (n=7) was used as a control. The brains of all animals were processed with a quantitative auto-radiography technique incubating the tissues with [^{3}H]flunitrazepam. The brain regions studied are mentioned above. Results were analyzed by a Student t test.

3. Results

3.1. Experiment 1

The effect of different doses of diazepam on the number of transitions in the exploratory behavior test, registered in mice with and without previous stressing, is shown in Fig. 1. Increase in the number of transitions (anxiolytic-like effect) apparently induced by forced swimming (Briones et al., 2002) was again observed in the previously stressed group (solid columns). Regarding diazepam, statistical analysis showed that stressed mice display different responses to this benzodiazepine agonist (right side); for stress condition F(1,90)=37.15, p<0.05; for drug treatment F(4, 90)=9.39, p<0.05; for interaction F(4,90)=12.33, p<0.05. Post hoc comparisons showed a clear increase in the number of transitions in intact mice after all doses of diazepam [(p<0.05) (left side)]. By contrast, not any dose enhanced the number of transitions when injected to stressed animals (right side). Data from this experiment demonstrate that the anxiolytic-like effect of diazepam is blocked when administered in stressed mice. Data from the activity test for all experimental groups did not show statistical differences (Table 1).

Table 1
Effect of several doses of diazepam on the number of transitions displayed by unstressed and stressed mice in the ambulatory behavior test

Treatment (mg/kg)	Ambulatory behavior (squares crossed/10 min)
No swimming	
0.00	78.8±6.3
0.125	93.0±7.2
0.25	105.1±6.4
0.50	87.2±8.4
1.00	101.3±7.8
Forced swimming	
0.00	118.6±11.5
0.125	101.2±8.3
0.25	87.6±8.5
0.50	110.2±8.7
1.00	103.1±7.5

Results are expressed as the mean±S.E. Two-way analysis of variance was nonsignificant in all cases (factor A; F(1,90)=0.77, factor B; F(4,90)=0.32, interaction AXB; F(4,90)=2.14).

3.2. Experiment 2

Results from this experiment are depicted in Fig. 2. Mice treated 24 h previously with allopregnanolone did not display behavioral changes in the anxiety test when compared with the control group. The known anxiolytic effect of diazepam was evident in mice previously injected with the lower dose of allopregnanolone (analysis of

Table 2
Effect of allopregnanolone or the combination allopregnanolone+diazepam on the ambulatory behavior of unstressed mice

Treatment (mg/kg)	Ambulatory behavior (squares crossed/10 min)
Control	74.9±6.2
Allopregnanolone 10.0	90.3±19.0
Allopregnanolone 20.0	72.9±7.2
Allopregnanolone 10.0+diazepam (0.5)	85.9±10.5
Allopregnanolone 20.0+diazepam (0.5)	80.2±7.2

Data are expressed as the mean±S.E. One-way analysis of variance showed no differences [F(4,45)=0.432; n.s.].

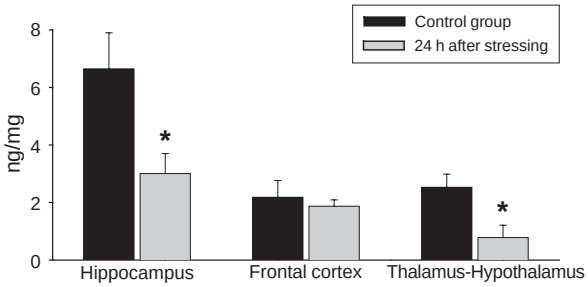

Fig. 3. GABA concentration in several brain areas of stressed and unstressed mice. The figure shows the mean±S.E. of GABA levels in 6 animals. Student *t* test, **p*<0.05.

variance $F(4, 45)=9.53$; $p<0.05$). In contrast, such an effect was not observed in animals treated 24 h previously with the higher dose of the neurosteroid (20 mg/kg). The number of squares crossed by all of these mice during the activity test was not altered after treatments and is depicted in Table 2.

3.3. Experiment 3

Fig. 3 summarizes the GABA levels in three specific brain areas 24 h after the stress session. Except for the frontal cortex, a clear reduction in GABA concentration at the hippocampus ($t=2.54$; $p<0.05$) and thalamus–hypothalamus ($t=2.78$; $p<0.05$) areas was observed in stressed mice, suggesting a role of stress on the availability of this neurotransmitter.

3.4. Experiment 4

Labeling of [^{3}H]flunitrazepam is represented in Fig. 4. A significant reduction of this parameter was detected at the hypothalamus ($t=2.71$; $p<0.05$) and amygdala ($t=2.19$; $p<0.05$) 24 h after submitting mice to forced swimming. The rest of the brain areas did not show changes when compared with the corresponding unstressed control group.

4. Discussion

In this study, forced swimming induced a blockage in the anxiolytic effect of diazepam. In a similar way, another report showed that this stressful factor attenuated the anti-seizure efficacy of flurazepam in the incremental electro convulsive shock procedure (Deutsch et al., 1990). Still another study showed that slight variations in the forced swimming procedure, such as the inclusion of cold-water, can decrease the anti-seizure ability of flurazepam and clonazepam (Weizman et al., 1989).

Several mechanisms underlying such effects were previously proposed. Two such mechanisms are: 1) The release of endogenous ligands with GABA-negative properties such as beta carbolines (Deutsch et al., 1990) that could counterbalance the inhibitory actions of benzodiazepines; and 2) The abrupt release of corticosterone during stress that supposedly alters the GABA synthesis and the GABA/benzodiazepine receptor expression (Weizman et al., 1989).

The $GABA_A$ receptor is composed of subunits belonging to subunit classes α, β, γ, δ and ρ (Luddens and Wisden, 1991; Cutting et al., 1991). However, the α subunits seem to be mainly responsible for the pharmacological diversity at this receptor (Pritchett and Seeburg, 1990; Luddens et al., 1991). At present, there are up to six different variants known for the α subunit (Wafford et al., 1996) and the combination $\alpha_1\beta_2\gamma_2$ represents the major adult isoform (McKernan and Whiting, 1996). On the other hand, it is well known that the reduced metabolite of progesterone allopregnanolone is released in the rat brain as a response to forced swimming (Purdy et al., 1991, 1992). This neurosteroid is able to modify the GABA–benzodiazepine receptor by increasing the α4 mRNA synthesis (Gulinello et al., 2001; Hsu et al., 2003). These findings suggest that the allopregnanolone release occurring after forced swimming could change the α subunit of the $GABA_A$ receptor and subsequently alter the affinity of this receptor to benzodiazepines. Accordingly, it has been found that this receptor containing α_4 subunits are insensitive to lorazepam (Wafford

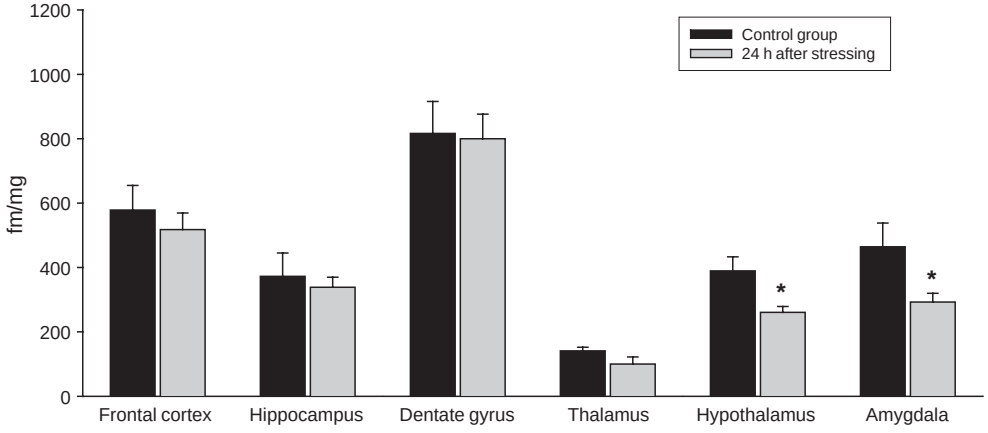

Fig. 4. Effect of forced swimming on [3H]flunitrazepam labeling in several brain regions. Each bar represents mean±S.E. of 7 mice. Student t test, **p*<0.05.

et al., 1996), diazepam (Wisden et al., 1991; Knoflach et al., 1996), pentobarbital and propofol (Wafford et al., 1996).

The current study shows that: 1) either forced swimming or a high dose of allopregnanolone is able to block the anxiolytic effect of diazepam, and 2) such an effect could not be blocked by a low dose of the neurosteroid. The first observation is similar to that of Gulinello et al. (2002), who found alterations in the anxiolytic profile of lorazepam after a subchronic treatment with allopregnanolone.

Overall, this evidence suggests that forced swimming produces conformational changes in the GABA–benzodiazepine complex that alter the pharmacological profile of benzodiazepines. However, this idea needs to be further explored, since it has been reported that such changes in the chemical composition of $GABA_A$ receptor have only been observed 48 h after a subchronic exposure to the steroid (Gulinello et al., 2001).

The decrease in GABA levels in the hippocampus and thalamus–hipothalamus of stressed mice is in agreement with the loss of the anxiolytic-like effect of diazepam, since the benzodiazepines act only in the presence of this neurotransmitter (Korpi et al., 2002). Accordingly, and under similar conditions of testing, we have found that forced swimming also is able to block the action of picrotoxin, a GABA-gated chloride ion channel blocker (Briones et al. 2002).

Other authors have found that GABA levels increase after exposure of animals to different stressful stimuli (Otero Losada, 1988; Acosta et al., 1993; Yoneda et al., 1983; Cook, 2004). These apparent discrepancies seem to be due mainly to the stressful factor used and the time at which the biochemical determination was made. While most biochemical analysis was done immediately after stressing, our determinations were made 24 h after swimming. Nevertheless, the idea that a brain GABA level decrease is accompanied by changes in the pharmacological response of agents acting through GABA/benzodiazepine receptor–chloride ionophore complex needs to be further explored.

Regarding $GABA_A$ receptor density, the most frequently studied brain areas influenced by stress are cerebral cortex, striatum, hippocampus, amygdala and hypothalamus (Drugan et al., 1989; Wilson and Biscarde, 1994; Stone et al., 2001). At the frontal cortex we did not find changes in the receptor density. According to other authors, under similar stress conditions the specific binding of [^{3}H] flunitrazepam to brain cortical membranes is not altered (Deutsch et al., 1994). On the other hand, we found a decrease in the $GABA_A$ receptor density in hypothalamus and amygdala, which could mean that these two brain regions are more sensitive to stress than the other areas studied. Along these lines, the amygdala has been closely related to emotional behavior, including fear, anxiety and aggression (Graeff et al., 1993; Pesold and Treit, 1994, 1995). Additionally, the hypothalamus is one of the brain regions responsible for controlling the hypothalamus–pituitary–adrenocortical axis (Cullinan et al., 1993) through the inhibition mediated by GABAergic cell groups (Bowers et al., 1998).

These data suggest that forced swimming induces modifications in the GABA tissue levels and in the GABA/benzodiazepine receptor density in a region specific manner, which could be the underlying reason for the lack of the anti-anxiety effect of diazepam observed in mice. These results emphasize the necessity of analyzing the use of benzodiazepines after traumatic events.

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