

# *Effects of Handling on Elevated plus-maze Behavior and [<sup>3</sup>H]Flunitrazepam Binding*

## Efeitos da Manipulação sobre Comportamento no Labirinto em Cruz Elevado e a Ligação de [<sup>3</sup>H]Flunitrazepam

Maria Carolina Velásquez <sup>a</sup>; Carlos Uribe <sup>a</sup>; Silvia Botelho <sup>a</sup>; José G. Ortiz <sup>b</sup>; Carlos Conde <sup>a</sup>, Carlos Tomaz <sup>c</sup>

### ABSTRACT

The Elevated Plus-Maze (EPM) is a widely used anxiety test based on the rat's natural aversion to open spaces. Handling before the first EPM exposure is thought to be an additional aversive stimulus. This study assessed the behavioral effects of acute handling (30 s) at different time intervals (0, 5 and 30 minutes) before the first EPM trial and the [<sup>3</sup>H]Flunitrazepam binding to amygdaloid complex membranes. Animals handled 5-min prior to the first EPM exposure increased the number of entries and time spent in the open arms of the EPM on the second trial and had reduced [<sup>3</sup>H]Flunitrazepam binding in the amygdaloid complex, indicating a reduction of GABA<sub>A</sub> transmission. These results suggest that acute handling, prior to an animal's first EPM exposure (5-min), may induce long-term behavioral and neurochemical changes on the GABA<sub>A</sub> system.

**KEY WORDS:** Acute handling; Elevated plus-maze; Anxiety; [<sup>3</sup>H]Flunitrazepam; GABA<sub>A</sub> receptor.

### RESUMO

O Labirinto em Cruz Elevado (LCE) é um teste de ansiedade amplamente utilizado e baseado na aversão natural dos ratos pelos espaços abertos. A manipulação dos animais antes da primeira exposição ao LCE poderia ser um estímulo aversivo adicional. No presente estudo foram avaliados os efeitos comportamentais da manipulação aguda (30 s) em diferentes intervalos de tempo (0, 5 e 30 minutos) antes da primeira exposição ao LCE, e a ligação de [<sup>3</sup>H]Flunitrazepam nas membranas do complexo amigdaloide.

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<sup>a</sup> Neuroscience and Behavior Group UIS-UPB, Department of Basic Sciences, Faculty of Health, Universidad Industrial de Santander. A.A. 678, Bucaramanga, Colombia.

<sup>b</sup> Department of Pharmacology and Toxicology, University of Puerto Rico, School of Medicine, PO Box 365067. San Juan, Puerto Rico 00936-5067.

<sup>c</sup> Laboratory of Neuroscience and Behavior, Department of Physiological Sciences, Institute of Biology, University of Brasilia, 70910-900 Brasilia-DF, Brazil.

Os animais manipulados 5-min antes da primeira exposição ao LCE aumentaram o número de entradas e tempo de permanência nos braços abertos do LCE na segunda sessão e mostraram diminuição da ligação de [<sup>3</sup>H]Flunitrazepam no complexo amigdaloide, indicando uma redução da transmissão de GABA<sub>A</sub>. Estes resultados sugerem que a manipulação aguda, antes da primeira exposição do animal ao LCE (5-min), pode induzir mudanças comportamentais e neuroquímicas no sistema GABAérgico a longo prazo no procedimento empregado neste estudo.

**PALAVRAS-CHAVE:** Manipulação aguda; Labirinto em Cruz Elevado; Ansiedade; [<sup>3</sup>H]Flunitrazepam; Receptor GABA<sub>A</sub>.

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## INTRODUCTION

The Elevated Plus-Maze (EPM) is a widely used anxiety test because its low cost and easy application. Its conceptual framework is based on the rats' natural aversion to open spaces and their preference to enclosed areas for housing and protection.<sup>9,16,17,22,26,34</sup>

The EPM test has characteristics that can evoke fear to the open arms, such as the height, open spaces and novelty.<sup>34</sup> The principal afferent pathways of open arms aversive information could be thigmotaxis and the visual pathways.<sup>8,20,23,33</sup>

In addition to the intrinsic EPM's aversive properties (open arms and height),<sup>34</sup> handling may also be a sensory stimulus affecting the animal's behavioral response.<sup>19</sup> Chronic handling prior to EPM testing failed to induce an anxiolytic-like effect, even with benzodiazepine or serotonergic drug treatments.<sup>1,7,13,27</sup> Thus handling, immediately before EPM testing, may be an important factor contributing to first-trial information acquisition and consolidation processes of long-term memory. Additionally, this kind of information has been found to dependent on the amygdala<sup>31,32</sup> and could be affected by the handling.

Handling and EPM exposure also appears to affect GABAergic transmission. Carvalho et al<sup>10</sup> showed that animals re-exposed to the EPM presents a significant decrease in dopamine and serotonin contents in amygdala and others structures related with fearful reactions. They demonstrated that exposure to a variety of stressful experiences results in important neurochemical changes.

Handling has been found to decrease binding of [<sup>3</sup>H]Flunitrazepam to cortical GABA<sub>A</sub> receptors in naïve-handled rats.<sup>2,3</sup> [<sup>3</sup>H]Flunitrazepam binding to GABA<sub>A</sub> receptors increases in amygdala and hippocampus of naïve-handled animals with a single EPM exposure.<sup>11</sup> In contrast, decreased GABA (K<sup>+</sup>-stimulated) release within the cortex and hippocampus of unhandled and of chronically-handled subjects re-exposed to the EPM with chlordiazepoxide treatment.<sup>27</sup>

This study evaluates the effects of different acute handling time intervals (0, 5 and 30 minutes) before the first EPM exposure and its effects on the long-term memory (second EPM exposure). We also determined [<sup>3</sup>H]Flunitrazepam binding in the amygdaloid complex to evaluate possible changes in GABA<sub>A</sub> receptors.

## MATERIALS AND METHODS

### Animals

Male Wistar rats (n=36), weighing 250±19 g, were obtained from the Faculty of Health, Universidad Industrial de Santander colony, Bucaramanga-Colombia. Rats were housed in groups of six/cage under controlled light/dark cycle (lights on at 07:00 hs, 12/12 hs), temperature (21±1°C) and humidity (65%±5). Food and water were available *ad libitum*. Testing was held between 14:00-18:00 hs. The control group (n=11) was housed in the same conditions; however, they were not exposed to the EPM. This study was approved by the Ethics Committee of the Faculty of Health of

Universidad Industrial de Santander (UIS), Bucaramanga, Colombia.

## Drugs

[<sup>3</sup>H]Flunitrazepam (85 Ci/mmol) was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO), while clonazepam from Roche Laboratories (Nutley, NJ).

## Elevated plus-maze (EPM)

The formica EPM, elevated 50 cm from the floor, consisted of two opposite open arms (OA), 50 x 12 cm with 1 cm high Plexiglas edge, which intersected two enclosed arms (EA), 50 x 12 x 40 cm. The intersection of the four arms (central platform) measured 12 x 12 cm. Animals were placed on the central platform facing an EA and were allowed to explore the maze freely during each 5-min trial. The maze was maintained under controlled conditions (300 lux in the central platform; 21°C; 65% humidity) and cleaned with alcohol (10% v/v) and dried before and between animal trials. Sessions were monitored and recorded via a video-camera system and behaviors scored using a semi-automated behavior analysis program Proxcom 3.20.<sup>13</sup> Arm entry and exit were defined as all four paws in or out of an arm, respectively. Decreased number of entries and the time spent in the OA were interpreted as an anxiogenic-like effect.<sup>16,26</sup>

## Behavioral Procedure

Effects of handling prior to first EPM exposure on behaviors observed during a second EPM trial: Thirty-six rats were used; 12 non-handled (tHE-0), 12 handled (during 30 seconds) 5 minutes (tHE-5) before the first EPM exposure, and 12 handled (during 30 seconds) 30 minutes before the first EPM trial (tHE-30). All animals were re-exposed to the EPM 72-h later, for 5 minutes without any handling.

## [<sup>3</sup>H]Flunitrazepam Binding

All animals were decapitated in a room adjacent to the maze immediately after the final EPM trial. The amygdaloid complex was dissected and

frozen at -70°C in tubes with buffer solution (50mM TRIS HCl, pH 7.4) for later binding assays. The tissue was homogenized (1:10 w/v) in ice-cold buffer and the homogenate was centrifuged twice at 2.500 g for 10-min. The resulting supernatant was centrifuged at 12.500 g for 20-min. Each pellet was then washed twice with buffer solution (1:10 w/v) and centrifuged at 12.500 g for 20-min. The pellets (crude synaptic membranes, P2) were re-suspended in ice-cold buffer solution, freeze-thawed at least three times, and stored at -80°C until assayed. Protein concentration was determined via Bradford assay<sup>6</sup> using bovine serum albumin (BSA) as standard.

The reaction was initiated by placing 20-24 µg of tissue-protein in tubes containing 2.0nM of [<sup>3</sup>H]Flunitrazepam in a final volume of 400µL of 50mM TRIS-HCl, pH 7.4.<sup>12</sup> Non-specific binding was determined in the presence of 10<sup>-4</sup>M clonazepam.<sup>25</sup> All samples were then incubated at 25°C for 4 min and 100µL triplicates of each group were rapidly filtered in Millipore AP 40 prefilters in a Millipore manifold, followed by two-2.5mL cold buffer washes. Radioactivity of the dried filters was quantified in a Beckman Coulter LS 6500 counter with 4mL of scintillation cocktail EcoLume (ICN).

## Statistical Analysis

The behavioral parameters measured were: number of entries, percentage of entries, time spent, percentage of the time in the Open Arms (OA) and Enclosed Arms (EA).<sup>16,26</sup> The data were analyzed using Two-way ANOVA test (F1: Exposure; F2: Handling), followed by *post hoc* Bonferroni test. For the [<sup>3</sup>H]Flunitrazepam binding assays, animals from the tHE-5 group were further divided in two groups: tHE-5(-), animals that did not enter the OA during the second trial (n=7); and tHE-5(+), animals that entered the OA during the second trial (n=5). One animal from the tHE-5 and from tHE-30 groups entered the OA and were excluded for binding assays. The specific binding data were analyzed via One-Way ANOVA test for between-group differences, followed by Bonferroni test for multiple

comparisons. For all analyses, statistical significance was set at  $p < 0.05$ .

## RESULTS

The effects on the entries and time in OA and EA are described in table 1. The analyses with Two-way ANOVA showed differences between sessions for the entries in EA ( $F(1,66) = 20.378$ ;  $P < 0.001$ ), but no significant difference between treatments ( $F(2,66) = 3.040$ ;  $P > 0.05$ ) and either for sessions-treatment interaction ( $F(2,66) = 0.019$ ;  $P > 0.05$ ). In the time into EA the test demonstrated differences attributable to session ( $F(1,66) = 27.089$ ;  $P < 0.05$ ) but no significant difference between session-treatment interactions ( $F(2,66) =$

$0.480$ ;  $P > 0.05$ ). The *post hoc* analysis revealed that in the first session the animals had fewer entries and time into the EA in comparison to the second one ( $P < 0.05$ ).

For the variables registered in the OA, Two-way ANOVA showed differences in the handling: entries ( $F(1,68) = 4.511$ ;  $P < 0.05$ ), time into ( $F(1,68) = 4.807$ ;  $P < 0.05$ ). Two-way ANOVA showed no significant difference for the sessions in the entries ( $F(1,68) = 0.450$ ;  $P > 0.05$ ) and time ( $F(1,68) = 0.355$ ;  $P > 0.05$ ); or interaction between sessions-treatment in entries ( $F(1,68) = 1.799$ ;  $P > 0.05$ ) and time ( $F(1,68) = 1.799$ ;  $P > 0.05$ ). *Post hoc* analysis revealed that the tHE-5 group had more entries and time into OA than tHE-0 group.

Table 1. Effects of different handling protocols on the behavior in first and second exposure of the EPM.

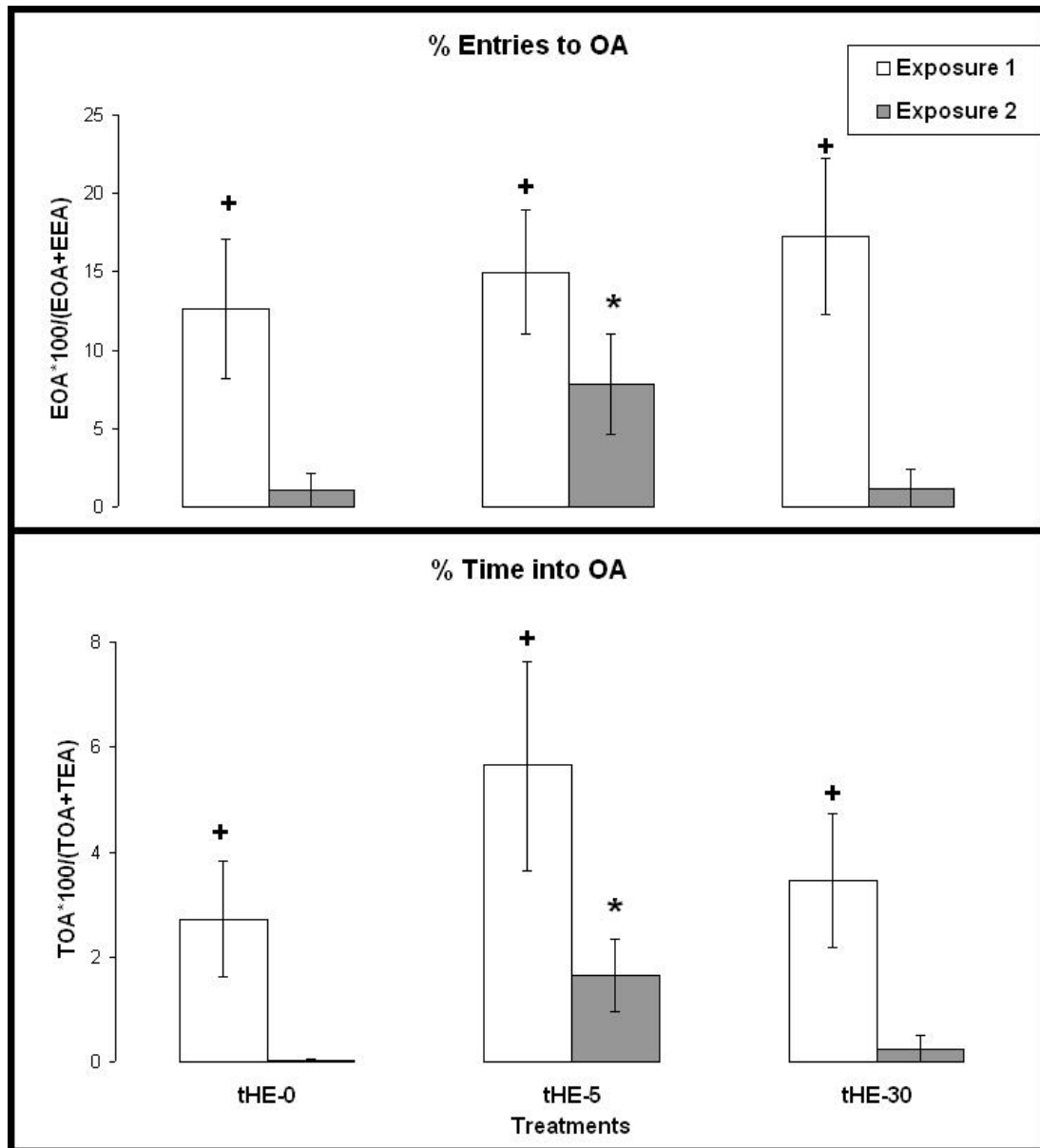
Parameter of Behavior	tHE-0		tHE-5		tHE-30	
	Average	±SEM	Average	±SEM	Average	±SEM
<b>Exposure 1</b>						
Entries to OA	1.25	0.48	2.00	0.70	1.33	0.47
Time into the OA	7.12	2.77	14.38	4.97	9.51	3.43
Entries to EA	6.42	1.03	7.83	1.06	5.50	0.97
Time into the EA	264.31	6.32	256.24	9.60	272.48	5.72
<b>Exposure 2</b>						
Entries to OA	0.08	0.08	0.66*	0.28	0.08	0.08
Time into the OA	0.06	0.07	4.46*	1.77	0.69	0.69
Entries to EA	3.08	0.79	4.42	0.84	2.42	0.54
Time into the EA	294.34	1.28	283.28	6.22	291.25	2.70

Legend: Mean  $\pm$  SEM. Animals without handling before the first exposure (tHE-0); animals handled 5 minutes before first exposure (tHE-5); animals handled 30 minutes before first exposure (tHE-30); Open Arms (OA); Enclosed Arms (EA).

\* $p < 0.05$  compared to tHE-0 (Bonferroni test).

The percentage of entries and time into the OA are illustrated in Figure 1. For these variables, the Two-way ANOVA showed significant difference for the handling: percentage of entries ( $F(1,68) = 5.885$ ;  $P < 0.05$ ), percentage of time ( $F(1,68) = 4.852$ ;  $P < 0.05$ ). There were no significant differences between sessions for the percentage of

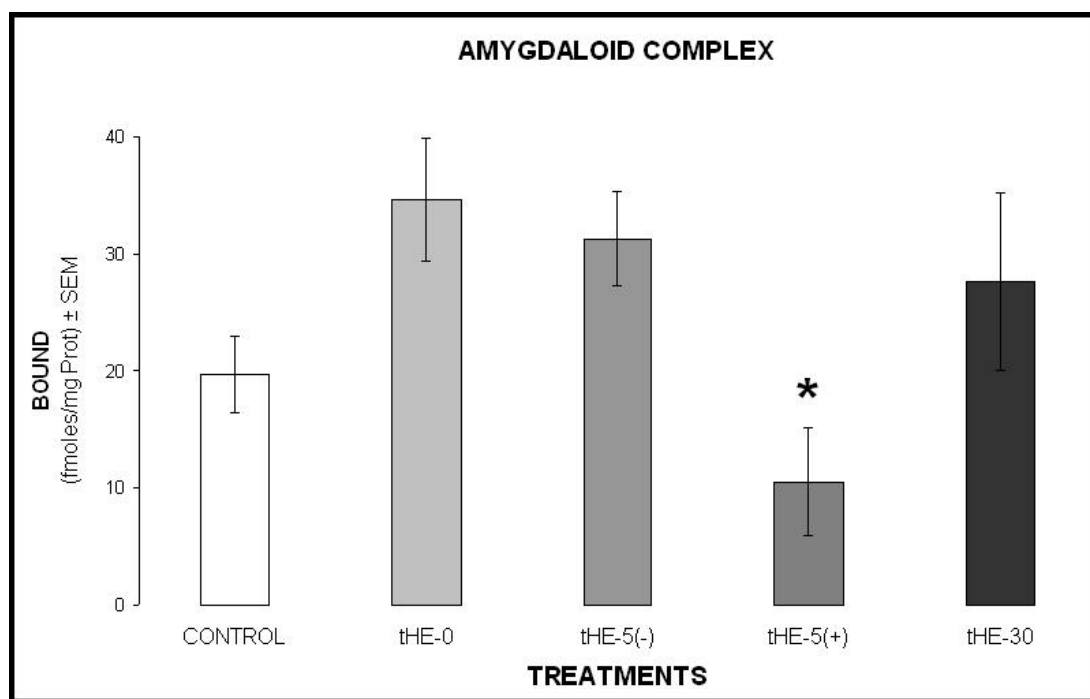
entries ( $F(1,68) = 0.046$ ;  $P > 0.05$ ), and percentage of time ( $F(1,68) = 0.400$ ;  $P > 0.05$ ), or session-treatment interactions: percentage of entries ( $F(1,68) = 2.653$ ;  $P > 0.05$ ), percentage of time ( $F(1,68) = 2.688$ ;  $P > 0.05$ ). *Post hoc* analysis revealed that the tHE-5 group had more percentage of entries and time into OA than the tHE-0 group.



**Figure 1.** Mean  $\pm$  SEM of the percent of the entries (top) and time (spent bottom) in the Open Arms after exposure 1 (white bars) and exposure 2 (grey bars) to the EPM in the different handling treatments. \*  $P < 0.05$  compared to tHE-0 group in the second exposure; +  $P < 0.05$  compared first to second session (Bonferroni test).

For [ $^3\text{H}$ ]Flunitrazepam binding assays (Figure 2), the tHE-5 group (5-min interval) was further divided in two additional groups; i.e. animals who did not enter the OA (tHE-5(-)) and those that did (tHE-5(+)) (see statistical analysis for details).

In the amygdaloid complex, specific binding in the tHE-5(+) group was significantly lower than in the tHE-0 group (One-way ANOVA  $F(4,18)=3.45$ ,  $P<0.05$ , followed by Bonferroni test ( $P<0.05$ )).



**Figure 2.** Specific [ $^3\text{H}$ ]Flunitrazepam binding to synaptic membranes of the amygdaloid complex in the different handling treatments. \*  $P<0.05$  compared to tHE-0 group (Bonferroni test).

## DISCUSSION

The behavior of rodents in the EPM is usually viewed as an interaction between open-space-related unconditioned stimuli, individual susceptibility and previous experience in this environment.<sup>4,5,14,17,18,22,26,34</sup> Aversive stimuli involved in EPM protocols have been more related to low brightness conditions and less related to thigmotaxis in the open arms and the visual pathways.<sup>8,20,23,33</sup> In addition to the intrinsic EPM's aversive properties (open arms and height)<sup>34</sup>, handling may also be a sensory stimulus affecting the animal's behavioral response.<sup>15</sup>

The effects of chronic and sub-chronic handling have been previously investigated in EPM,<sup>1,7</sup> although few studies have explored the influence of acute handling.<sup>2,3</sup> It has been observed that the animal experience on the EPM, taking this like a stressful event, can produce changes in the GABAergic system, but also in the serotonergic and dopaminergic systems.<sup>10</sup>

Andrews et al.<sup>2,3</sup> showed a lower open arm entries in non-handled animals on the first exposure. However, Lapin<sup>24</sup> reported no differences in a 5-minute exposure to the EPM between non-handled and handled animals for 21 days. This results are in agreement with ours showing that handling, a few minutes before EPM, did not influence the behavior

during the first exposure. Thus, the acquisition process related to the OA information appears to be unaltered. However, it can cause long-term behavioral and neurochemical changes.

On the other hand, the 5-min handling-testing interval (tHE-5), employed in this study may have been too short. However, some animals handled 5-min prior to their first EPM trial (tHE-5(+)) increased the number of entries and time spent in the OA during the second trial suggesting an alteration on the mnemonic process, resulting in a possible aversive spaces (OA) amnesia, and in re-exploration to made re-acquisition of this information.

The amygdaloid complex, in particular the basolateral nucleus, is known to have high GABA<sub>A</sub>-receptor concentrations and is involved in the amnesic effects of some drugs such as diazepam.<sup>28,29,30</sup> Moreover, this structure is considered important for the aversive stimuli evaluation, being more involved in moderate-intensity stimuli than in high-intensity and clearly aversive situations.<sup>7,29</sup> Animals with an alteration on the OA evocation and an increase of the exploration during the second trial (tHE-5(+)), had diminished in specific binding for GABA<sub>A</sub> receptors in the amygdaloid complex, indicating a decreased GABA<sub>A</sub> transmission. Conversely, rats handled 5-min prior to their first EPM trial that did not enter the OA during their second trial (tHE-5(-)), show increased specific [<sup>3</sup>H]Flunitrazepam binding. In addition, animals without any previous handling (tHE-0) or with distant handling (tHE-30) demonstrated few or no OA entries during their second EPM exposure and increased in the specific binding for GABA<sub>A</sub> receptors. Together these results suggest that avoidance of the OA on the second EPM exposure increases GABAergic transmission in the studied regions.

Reduced [<sup>3</sup>H]Flunitrazepam binding is observed in amygdaloid complex of animals that did not show avoidance to the OA (tHE-5(+)). Such reduction could reflect internalized GABA<sub>A</sub> receptors, excluded from our membrane prepara-

tion. Alternatively, the large time interval between the handling prior to the first exposure and the tissue extraction for the [<sup>3</sup>H]Flunitrazepam binding (after the second EPM trial), does not preclude the possibility that GABA<sub>A</sub> receptors were being replaced by others containing alpha-4 or alpha-6 subunits that do not bind [<sup>3</sup>H]Flunitrazepam.<sup>21,35</sup> On the other hand, the GABAergic system may have been exerting its effects on other regions, such as the hippocampus, that in turn could have influenced memory and anxiety related processes.

In conclusion, these results suggest that handling immediately before the first EPM exposure may be an important variable influencing long-term memory formation (specially the aversive memory). These effects are dependent on the interval between handling and EPM exposure. Furthermore, handling not only induces complex long-term behavioral changes, but also causes neurochemical changes, that may contribute to the decreased [<sup>3</sup>H]Flunitrazepam binding in the amygdaloid complex.

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