

## Corticosterone does not change open elevated plus maze-induced antinociception in mice

Joyce Mendes-Gomes<sup>a,b</sup>, Tarciso Tadeu Miguel<sup>b,c</sup>,  
Vanessa Cristiane Santana Amaral<sup>b,c,d</sup>, Ricardo Luiz Nunes-de-Souza<sup>a,b,c,\*</sup>

<sup>a</sup> Programa de Pós-Graduação em Psicobiologia, FFCLRP – USP Av. Bandeirantes, 3900, CEP 14040-901 Ribeirão Preto, SP, Brazil

<sup>b</sup> Lab. Neuropsicofarmacologia, Faculdade de Ciências Farmacêuticas – UNESP Rod. Araraquara-Jaú, Km 01, CEP 14801-902 Araraquara, SP, Brazil

<sup>c</sup> Programa Interinstitucional de Pós-Graduação em Ciências Fisiológicas, UFSCAR-UNESP Rod. Washington Luis, Km 235, CEP 13565-905 São Carlos, SP, Brazil

<sup>d</sup> Unidade Universitária de Ciências Exatas e Tecnológicas, UnUCET-UEG Br 153 n.3105 CEP: 75132-903 Anápolis, GO, Brazil

### ARTICLE INFO

#### Article history:

Received 21 February 2011

Revised 30 June 2011

Accepted 7 July 2011

Available online 20 July 2011

#### Keywords:

Anxiety  
Fear  
Different types of EPM  
Antinociception  
Formalin test  
Corticosterone  
Adrenalectomy  
Mice

### ABSTRACT

It has been demonstrated that the exposure of rodents to the standard elevated plus-maze (sEPM: 2 open and 2 enclosed arms) elicits defensive behavioral reactions and antinociception and also activates the hypothalamo-pituitary-adrenal (HPA) axis. We have recently reported that EPM-induced antinociception is particularly observed when rats and mice are exposed to a totally open EPM (oEPM: 4 open arms). Given that the oEPM seems to be a more aversive situation than the sEPM, we hypothesized that oEPM exposure would induce higher plasma levels of corticosterone than sEPM exposure in mice. In this study, we investigated the influence of exposure to eEPM (enclosed EPM: 4 enclosed arms), sEPM or oEPM on plasma corticosterone levels in mice, with or without prior nociceptive stimulation (2.5% formalin injection into the right hind paw). We also tested whether the nociceptive response in the formalin test and oEPM-induced antinociception are altered by adrenalectomy. Results showed that oEPM-exposed mice spent less time licking the injected paw than sEPM- and eEPM-exposed animals. All three types of EPM exposure increased plasma corticosterone when compared to the basal group, but sEPM- and oEPM-exposed mice showed higher corticosterone levels than eEPM-exposed mice. Prior nociceptive stimulation (formalin injection) did not enhance the plasma corticosterone response induced by the three types of EPM exposure. Indeed, formalin injection appeared to provoke a ceiling effect on plasma corticosterone concentration. Furthermore, neither the nociceptive response in the formalin test nor oEPM-induced antinociception was changed by adrenalectomy. Present results suggest that oEPM antinociception does not depend on corticosterone release in mice.

© 2011 Elsevier Inc. All rights reserved.

### Introduction

Threatening or aversive events can activate the hypothalamo-pituitary-adrenocortical (HPA) axis and consequently, stimulate the secretion of glucocorticoids from the adrenal cortex (e.g., Marin et al., 2007). Glucocorticoids, especially cortisol in primates and corticosterone in rodents, act on the expression and regulation of genes throughout the body and affect many physiological process, that prepare the organism for the changes in energy and metabolism required when it is coping with stressful situations (Akil and Morano, 1995; Levine, 2005; Marin et al., 2007). Therefore, serum corticosterone level is widely accepted as a biological marker of stress (Herman et al., 2005; Korte, 2001).

\* Corresponding author at: Lab. Farmacologia, Faculdade de Ciências Farmacêuticas – UNESP Rod. Araraquara-Jaú, Km 01, CEP 14801-902 Araraquara, SP, Brazil. Fax: + 55 16 3301 6980.

E-mail addresses: [souzarn@cfar.unesp.br](mailto:souzarn@cfar.unesp.br), [ricardo.souza@pq.cnpq.br](mailto:ricardo.souza@pq.cnpq.br) (R.L. Nunes-de-Souza).

In general, activation of the HPA axis also attenuates pain sensitivity (Lariviere and Melzack, 2000; Vissers et al., 2004; Yarushkina, 2008; Yokoro et al., 2003). For instance, systemic administration of glucocorticoids at physiological doses, so as to produce an increase in blood hormones comparable to those seen in a stressful situation, leads to reduction in the nociceptive response in rats (Yarushkina, 2008). However, Yarushkina (2008) also emphasized a linear correlation between the corticosterone level and the pain threshold, demonstrating that the blockade of HPA function with an intra-hypothalamus injection of the exogenous corticoid dexamethasone (at pharmacological doses) completely eliminated the stress-induced analgesia. The same pattern of results was observed on surgical removal of adrenal glands (ADX: adrenalectomy) (Yarushkina, 2008). Nevertheless, it is important to point out that the observed effects of ADX on pain response are ambiguous, since, depending on the nature of the pain stimulus, adrenal removal can increase (Yokoro et al., 2003) or decrease nociceptive behavior (Vissers et al., 2004).

It has been demonstrated that the exposure of rodents to the standard elevated plus-maze (sEPM: with two open and two

enclosed arms), an animal model of anxiety (Handley and Mithani, 1984; Lister, 1987; Pellow et al., 1985), activates the HPA axis, leading to an enhancement of plasma corticosterone (Rodgers et al., 1999). The EPM test is based on the natural fear of rodents to open spaces (Handley and Mithani, 1984; Lister, 1987; Pellow et al., 1985). Animals exposed to the EPM display at least two main strategies: they 1) avoid the open arms and 2) escape from an open arm to enter a safer, closed arm (Graeff et al., 1993; Pinheiro et al., 2007). In addition, Lee and Rodgers (1990) demonstrated that mice exposed to the EPM not only display defensive behavioral reactions but also antinociception. In experiments carried out in our laboratory, sEPM-exposed mice do not show marked antinociception, assessed with the formalin nociception test. Nevertheless, when mice are placed in a totally open elevated plus-maze (oEPM: with four open arms) a marked reduction in the time spent licking the formalin injected paw (i.e., antinociceptive response) is observed (Mendes-Gomes et al., 2011; Mendes-Gomes and Nunes-de-Souza, 2005, 2009). Given that the animals cannot avoid or escape the highly aversive open arms of the oEPM, this apparatus seems to be a more aversive situation than the sEPM. Therefore, we hypothesized that oEPM-exposed animals would exhibit a higher level of corticosterone release than sEPM- and eEPM-exposed mice. If that hypothesis is correct, oEPM-induced antinociception (Mendes-Gomes and Nunes-de-Souza, 2005, 2009; Mendes-Gomes et al., 2011) might be mediated by the release of corticosterone.

Interestingly, the well-known anxiolytic-like effect produced by benzodiazepine injections into the dorsal portion of the midbrain periaqueductal gray matter (dPAG) (Mendes-Gomes and Nunes-de-Souza, 2005; Russo et al., 1993) was not observed in EPM-exposed animals that had received a formalin injection (a nociceptive stimulus) into the hind paw (Mendes-Gomes and Nunes-de-Souza, 2005). A similar result has also been obtained with chemical lesion of the dPAG (Mendes-Gomes and Nunes-de-Souza, 2009). Importantly, in both cases, a clear antianxiety effect was observed in mice that had not received the prior injection of formalin into the hind paw (Mendes-Gomes and Nunes-de-Souza, 2005, 2009). Since pain is an aversive stimulus, it possibly induces the release of corticosterone, which in turn, may activate limbic brain structures [e.g., amygdala (Carrasquillo and Gereau, 2007; Ji et al., 2007; Neugebauer et al., 2004; Tanimoto et al., 2003) and anterior hypothalamus (Lumb, 2004)] responsible for the mediation of defensive behaviors (e.g., avoidance of the open arms in the oEPM). In view of these findings, in the present study we analyze the effects of exposure to the enclosed, standard or open EPM on the release of corticosterone in animals subjected or not subjected to the nociceptive formalin test. In addition, we analyzed whether the nociceptive response in the formalin test and oEPM-induced antinociception are affected by adrenalectomy.

## Materials and methods

### Subjects

The subjects were male Swiss mice (from Univ. Estadual Paulista - UNESP, SP, Brazil), weighing 25–35 g. They were housed in groups of 7 per cage (41 cm × 34 cm × 16 cm) and maintained under a 12:12 h light/dark cycle (lights on at 7:00 a.m.) in a temperature (23 ± 1 °C) and humidity (55 ± 5%) controlled environment. Food and drinking water were freely available. All mice were experimentally naïve, and experimental sessions were carried out during the light phase of the cycle (9 a.m.–5 p.m.).

### Formalin test

Nociception was assessed by the formalin test as previously described (Abbott et al., 1995). The formalin test causes a two-phase nociceptive response (Dubuisson and Dennis, 1977). The first phase

begins immediately after formalin injection and lasts approximately 5 min. It results from the direct stimulation of nociceptors (Dubuisson and Dennis, 1977; McCall et al., 1996). The second phase begins 20 min after the injection and lasts approximately 40 min (Bon et al., 2002). This phase is caused by C-fiber activation (McCall et al., 1996; Tjolsen et al., 1992) and also involves a period of sensitization during which inflammatory phenomena occur (Le Bars et al., 2001; Tjolsen et al., 1992). In response to formalin injection into the paw, animals exhibit pain- or recuperative-related behaviors as lifting/guarding and licking and biting (Bon et al., 2002; Dubuisson and Dennis, 1977; Sufka et al., 1998). Nevertheless, in the present study only the time spent licking the formalin-injected paw was used as nociceptive response.

### Apparatus

The basic elevated plus-maze design was similar to that originally validated for mice (Lister, 1987). The standard EPM (sEPM) consisted of two open arms (30 cm × 5 cm × 0.25 cm) and two enclosed arms (30 cm × 5 cm × 15 cm) connected to a common central platform (5 cm × 5 cm). The apparatus was constructed from wood (floor) and transparent glass (clear walls) and was raised to a height of 38.5 cm above floor level. The other two mazes were similarly constructed, but comprised either four enclosed arms (eEPM) or four open arms (oEPM).

On the sEPM, mice were individually placed on the central platform of the maze facing the left open arm. Both the eEPM and oEPM were similarly positioned in the experimental room and the experimenter placed the animal facing the arm that corresponded in direction to the sEPM left open arm, even though the eEPM had no open arms and the oEPM no enclosed arms. Between subjects, the mazes were cleaned thoroughly with 20% ethanol and a dry cloth. All sessions were video-recorded by a camera linked to a monitor and DVD in the adjacent laboratory.

### Corticosterone radioimmunoassay

The radioimmunoassay for corticosterone was conducted as described previously by our research group (Marin et al., 2007). The assay was performed with antibodies from Sigma (St. Louis, MO) and (3H)-corticosterone from New England Nuclear (Boston, MA). The method was adapted from that described by Sarnyai et al. (1992). Briefly, 20 µl of plasma was diluted 50 times with 0.01 M PBS and placed in a water bath at 75 °C for 1 h to heat-inactivate the corticosteroid-binding globulin. Aliquots of 100 µl of a solution of antibody and (3H)-corticosterone (10,000 to 20,000 cpm/ml) were added to each sample, mixed and incubated overnight at 4 °C. Dextran-coated charcoal was used to adsorb free steroid after incubation. Tubes were centrifuged at 2000 ×g for 10 min at 4 °C, and the supernatant from each tube was transferred to a scintillation vial. Radioactivity was measured by liquid scintillation spectrometry. Standard curves were constructed using 31.25, 62.5, 125, 250, 500 and 1000 pg/100 µl (triplicates) of corticosterone (Sigma). After dilution, all the concentrations of the corticosterone samples were within the linear range of the standard curve. The lower limit of detection was 0.23 µg/dl and inter- and intra-assay variations were 4.0% and 8.1%, respectively.

### Procedures

#### *Experiment 1: Corticosterone levels in mice exposed to different types of elevated plus-maze: influence of nociceptive stimulation*

Swiss male mice (n = 6–9/group) were transported to the experimental room and left undisturbed for at least 60 min prior to testing. In half of the animals, 50 µl of 2.5% formaldehyde solution was injected into the dorsal surface of the right hind paw and the mouse was immediately placed in an individual glass holding cage (30 cm × 20 cm × 25 cm). After an interval of 25 min, each animal was exposed to the eEPM, sEPM

or oEPM ( $n=6/\text{group}$ ), where the time (in seconds) spent licking the injected paw was noted over a period of 10 min (25–35 min after formalin injection). In order to investigate whether corticosterone levels are changed by nociceptive stimulation, the other half of the mice were subjected to a similar procedure, except that they did not receive the formalin injection (eEPM, sEPM and oEPM,  $n=8, 9$  and  $6$ , respectively). It is important to highlight that all animals were randomly distributed between the groups.

After EPM exposure, each mouse was individually placed in a cage lined with its cage bedding and left undisturbed for 5 min. This time was based on work in our laboratory demonstrating that a peak in corticosterone secretion occurs 5 to 10 min after exposure to two different anxiety/fear tests, the rat exposure test (Amaral et al., 2010) and the sEPM (personal communication, Karina Santos Gomes). The animals were then rapidly transported to an adjacent room where they were decapitated. Trunk blood was collected in heparinized tubes and centrifuged at  $2000 \times g$  for 10 min, at  $4^\circ\text{C}$ . The plasma was stored at  $-80^\circ\text{C}$  until the radioimmunoassay. An additional group composed by experimentally naïve mice (corticosterone basal group,  $n=5$ ) was placed in individual home cages and followed the same protocol.

#### Experiment 2: Influence of adrenalectomy on oEPM-induced antinociception

Twenty-four mice were anesthetized with i.p. injected ketamine (100 mg/kg) and xylazine (10 mg/kg) and both adrenal glands were removed (adrenalectomy, ADX) by a single dorsal midline incision in the skin at the level of the kidneys. Sham surgery consisted of a single dorsal midline incision where the adrenal glands were located but not removed. All mice (ADX and sham) were given free access to water and 0.9% NaCl drinking solution, as well as food.

Five days after the ADX or sham procedure, each mouse was injected with formalin as described above. After that they were exposed to the glass box and the time spent licking the paw was recorded for 5 min (Phase 1 of the formalin test). Twenty-five minutes after formalin injection, animals were exposed to the oEPM or eEPM ( $n=6/\text{group}$ ) and the nociceptive response was recorded for 10 min.

#### Behavioral analysis

Videotapes were scored by a highly trained observer using an ethological analysis software package developed by Dr. Morato's group at Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, USP (Brazil). In addition to recording the time spent licking the formalin-injected paw (see above), the total number of arm entries (arm entry = all four paws into an arm) was scored. For groups exposed to the sEPM, the frequency of enclosed and open arm entries, % open arm  $[(\text{open}/\text{total}) \times 100]$  and % open arm time  $[(\text{open time}/300) \times 100]$  were also recorded. Although the duration of the test was 10 min, the data on exploration of the maze were calculated only during the standard (Carobrez and Bertoglio, 2005; Lister, 1987; Pellow et al., 1985; Rodgers and Johnson, 1995) first 5 min of the test.

#### Statistics

All results were initially submitted to Levene's test for homogeneity of variance. When appropriate, the data were transformed to the square root or log and then analyzed by Student's *t*-test for unrelated samples, one- or two-way analysis of variance (ANOVA) for independent factors [one-way (effect of nociception or of surgery type); two-way (factor 1: maze type, factor 2: nociception)]. Non-parametric data were analyzed by the Mann–Whitney *U* test. Where indicated by significant *F* values, group differences were identified by Duncan's test. A *P*-value  $\leq 0.05$  was required for significance.

#### Ethics

The experiments carried out in this study comply with the norms of the Brazilian Neuroscience and Behavior Society (SBNeC), based on the US National Institutes of Health Guide for the Care and Use of Laboratory Animals. Furthermore, all experimental protocols were analyzed and approved by the local Research Ethics Committee (CEP/FCF/Car. protocols 20/2005 and 43/2008).

## Results

#### Behavioral results

Fig. 1 illustrates the plasma corticosterone ( $\mu\text{g}/\text{dl}$ ) levels of mice in the control (basal: animals that were neither exposed to any EPM nor injected with formalin), eEPM-, sEPM- or oEPM-exposed groups that had or had not received a prior formalin injection into the hind paw. One-way ANOVA demonstrated a significant effect of exposure on corticosterone levels [ $F(6,39) = 10.44$ ;  $P < 0.001$ ]. Posterior comparisons revealed that all treated groups secreted more corticosterone than the control basal group. Moreover, the animals not subjected to the nociceptive test but exposed to the sEPM or oEPM showed higher levels of this glucocorticoid than the eEPM-exposed mice. Also, mice exposed to the eEPM and submitted to the formalin test showed higher levels of corticosterone when compared to that not subjected to the nociceptive test.

Table 1 shows the time spent licking the paw injected with formalin, by mice exposed to the eEPM, sEPM and oEPM. One-way ANOVA followed by Duncan's test revealed that oEPM-exposed mice showed a significantly lower nociceptive response than animals exposed to the eEPM and sEPM [ $F(2,15) = 12.02$ ;  $P < 0.01$ ].

Fig. 2 shows that formalin injection changed neither anxiety indices nor the locomotor activity during the first five minutes of

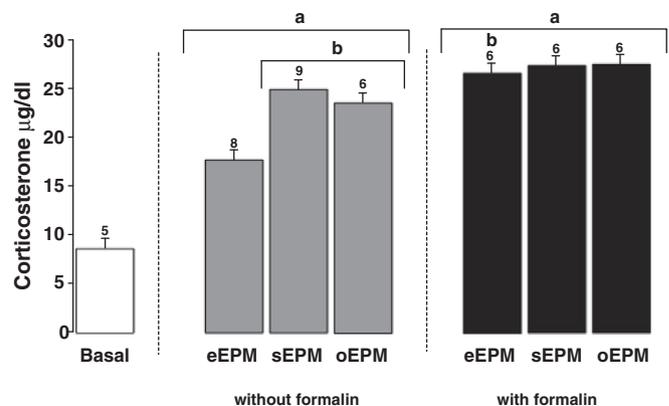


Fig. 1. Plasma levels of corticosterone in mice stimulated or not with formalin injection into the dorsal right hind paw and exposed to three types of mazes (eEPM, sEPM or oEPM);  $n=5-9/\text{group}$ . Numbers on the top of each bar represent the respective sample size. <sup>a,b</sup> $p < 0.05$  versus basal and without formalin eEPM groups, respectively.

Table 1

Effects of exposure to different types of mazes (eEPM, sEPM or oEPM) on total time spent licking (s) the formalin-injected paw recorded for 10 min during the tonic phase of this nociceptive test in mice.

Exposure	Time spent licking (s)
eEPM	$111.5 \pm 33.2$
sEPM	$99.4 \pm 23.8$
oEPM	$7.4 \pm 4.7^a$

$n=6/\text{group}$ .

<sup>a</sup>  $P < 0.01$  versus eEPM and sEPM.

exposure to the sEPM, relative to groups not subjected to this nociceptive test [(% open arm entries:  $t(12) = 1.13$ ;  $P = 0.28$ ; % open arm time:  $t(12) = 0.76$ ;  $P = 0.46$  and frequency of enclosed arm entries:  $t(12) = -0.03$ ;  $P = 0.97$ ].

Table 2 shows the total mean numbers of arm entries and time (in seconds) spent on the central platform during the first 5 min of exposure to the eEPM, sEPM or oEPM in mice with or without prior formalin injection into the right hind paw. Regarding total arm entries, two-way ANOVA revealed significant changes for the maze type factor [ $F(2,33) = 31.33$ ;  $P < 0.001$ ], but not for the nociception factor [ $F(1,33) = 2.79$ ;  $P = 0.10$ ] or maze type  $\times$  nociception interaction [ $F(2,33) = 0.25$ ;  $P = 0.78$ ]. Post-hoc comparisons revealed that animals exposed to the sEPM or oEPM exhibited lower frequencies of arm entries than the corresponding groups exposed to the eEPM. The non-parametric Mann–Whitney  $U$  test, applied to the time spent on the central square, revealed that sEPM-exposed animals spent more time on the square than eEPM-exposed mice, an effect that did not

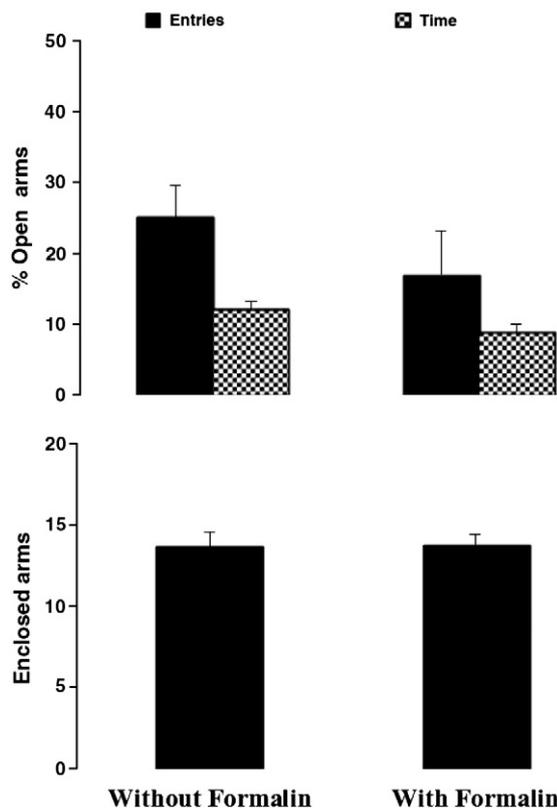


Fig. 2. Lack of effect of formalin injection into the dorsal right hind paw (nociceptive stimulation) on percentages of entries or percentage of open arm time (OA) and frequency of enclosed arm entries (EE) in the sEPM in mice;  $n = 6$ –8/group.

Table 2

Total arm entries and time spent in the central square of the enclosed, standard and open EPM (eEPM, sEPM and oEPM) exhibited by mice tested or not with formalin injection into the dorsal right hind paw.

Exposure	Formalin	Behavior	
		Total arm entries	Center time
eEPM	Without	32.4 $\pm$ 2.4	72.3 $\pm$ 6.2
sEPM		18.4 $\pm$ 1.2 <sup>a</sup>	129.9 $\pm$ 12.8 <sup>ab</sup>
oEPM		17.7 $\pm$ 0.5 <sup>a</sup>	74.8 $\pm$ 8.1
eEPM	With	28.3 $\pm$ 2.1	83.2 $\pm$ 7.8
sEPM		17.0 $\pm$ 1.8 <sup>a</sup>	137.7 $\pm$ 11.9 <sup>a</sup>
oEPM		15.2 $\pm$ 3.0 <sup>a</sup>	100.0 $\pm$ 25.1

$n = 6$ –8/group. <sup>a</sup> $P < 0.05$  versus eEPM and oEPM, respectively.

depend on prior formalin injection (without formalin:  $U = 5$ ;  $Z = -2.66$ ;  $P < 0.05$ ; with formalin:  $U = 1$ ;  $Z = -2.72$ ;  $P < 0.05$ ). Moreover, sEPM-exposed mice that had not received the formalin injection spent more time on the center square than oEPM-exposed mice ( $U = 5$ ;  $Z = 2.45$ ;  $P < 0.05$ ). Additionally, the Mann–Whitney  $U$  test did not show any effect of formalin injection on time spent on the center square [oEPM ( $U = 14$ ;  $Z = -1$ ;  $P = 0.32$ ), sEPM ( $U = 21$ ;  $Z = -0.39$ ;  $P = 0.70$ ) or eEPM ( $U = 16$ ;  $Z = -0.32$ ;  $P = 0.75$ )].

Fig. 3 shows the lack of effect of adrenalectomy (ADX) on time spent licking the formalin-injected paw in mice during Phase 1 (0–5 min) and Phase 2 (25–35 min) of the nociceptive test. Phase 1 was analyzed while the mice were in the glass holding cage (GC) and Phase 2 was recorded during the exposure to the enclosed (eEPM) or open (oEPM) mazes. Student's  $t$ -test for unrelated samples revealed that ADX did not change the nociceptive response ( $t(22) = -1.01$ ;  $P = 0.32$ ) during Phase 1. Two-way ANOVA for Phase 2 revealed significant changes for maze type factor [ $F(1,20) = 19.25$ ;  $P < 0.001$ ], but not for surgery factor [ $F(1,20) = 0.92$ ;  $P = 0.35$ ] or maze type versus surgery interactions [ $F(1,20) = 0.03$ ;  $P = 0.87$ ]. Post-hoc comparisons revealed that oEPM-exposed mice spent less time licking the injected paw than eEPM-exposed mice.

Table 3 illustrates the effects of ADX on total arm entries and time (in seconds) spent on the central platform by mice exposed to the eEPM or oEPM for 5 min. The Mann–Whitney  $U$  test revealed that ADX did not alter the frequency of arm entries in mice exposed the two types of EPM (eEPM:  $U = 10.5$ ;  $Z = 1.2$ ;  $P = 0.23$ ; oEPM:  $U = 17.5$ ;  $Z = -0.08$ ;  $P = 0.94$ ). Nevertheless, the Mann–Whitney test revealed that oEPM-exposed mice showed a lower frequency of arm entries than eEPM, in sham groups alone (sham:  $U = 3.5$ ;  $Z = 2.33$ ;  $P < 0.05$ ; ADX:  $U = 11.0$ ;

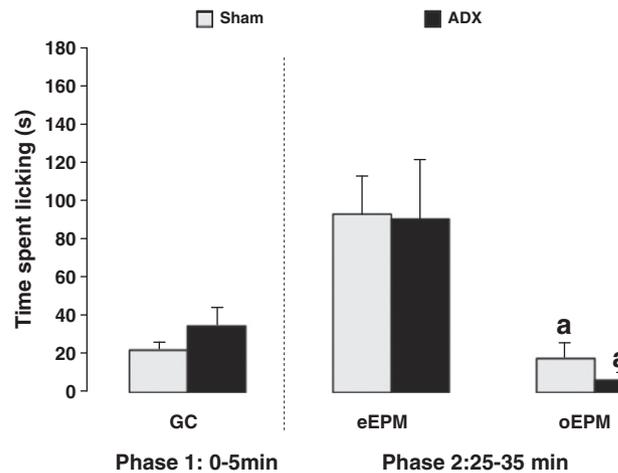


Fig. 3. Lack of effects of adrenalectomy (ADX) on time spent licking (s) the paw injected with 2.5% formalin, recorded during Phase 1 (0–5 min) or Phase 2 (25–35 min) of the nociceptive test. Phase 1 was analyzed while the mice were in the glass holding cage (GC) and Phase 2 was recorded during the exposure to the eEPM or oEPM;  $n = 6$ /group. <sup>a</sup> $P < 0.001$  versus eEPM.

Table 3

Lack of effects of adrenalectomy (ADX) on total arm entries and time spent on the central square of the enclosed and open EPM (eEPM, sEPM and oEPM) exhibited by mice stimulated with formalin injection into the dorsal right hind paw.

Exposure	Surgery	Behavior	
		Total arm entries	Center time
eEPM	Sham	21.3 $\pm$ 2.2	65.5 $\pm$ 5.5
oEPM		12.5 $\pm$ 2.0 <sup>a</sup>	123.5 $\pm$ 16.9 <sup>a</sup>
eEPM	ADX	15.5 $\pm$ 3.8	65.7 $\pm$ 18.0
oEPM		11.3 $\pm$ 1.4	134.3 $\pm$ 10.2 <sup>a</sup>

$n = 6$ /group.

<sup>a</sup>  $P < 0.05$  versus eEPM.

$Z = 1.12$ ;  $P = 0.26$ ). For the time spent on the central square, two-way ANOVA revealed significant changes for the maze type factor [ $F(1,20) = 21.50$ ;  $P < 0.001$ ], but not for the surgery factor [ $F(1,20) = 0.16$ ;  $P = 0.69$ ] or maze type  $\times$  surgery interactions [ $F(1,20) = 0.15$ ;  $P = 0.70$ ]. Post-hoc comparisons revealed that oEPM-exposed mice spent more time on the center square than corresponding groups exposed to the eEPM.

## Discussion

Present results demonstrated that the exposure of mice to any of three different types of EPM (eEPM, sEPM and oEPM) increases plasma corticosterone. This effect was more prominent when animals were exposed to a more aversive situation (e.g., sEPM and oEPM). Furthermore, formalin injection into the hind paw caused a high release of plasma corticosterone in mice exposed to the eEPM, sEPM or oEPM. In addition, the hypothesis that high levels of plasma corticosterone induced by oEPM exposure would modulate the oEPM-induced antinociception was not confirmed, since ADX did not alter the antinociceptive response of mice exposed to this aversive situation.

We have recently demonstrated that the anxiolytic-like effects produced by intra-PAG injection of midazolam (Mendes-Gomes and Nunes-de-Souza, 2005), or by bilateral lesion of the dPAG (Mendes-Gomes and Nunes-de-Souza, 2009), are abolished by concurrent nociceptive stimulation in mice. However, it remains unclear how nociceptive stimulation alters the antianxiety effects of these procedures. Considering that formalin injection causes an inflammatory response and, as a typical stressor, increases plasma ACTH and corticosterone (Taylor et al., 1998) which, in turn, may increase anxiety (Steimer and Driscoll, 2003), we have hypothesized that the high plasma levels of corticosterone induced by formalin injection could contribute to the antinociceptive effect observed in oEPM-exposed mice. In this context, we investigated in this study whether mice exposed to the eEPM, sEPM or oEPM, with or without a prior formalin injection, would show differences in plasma corticosterone levels. As shown in Fig. 1, irrespective of the nociceptive stimulus they had received, sEPM- and oEPM-exposed mice showed increased plasma levels of corticosterone, relative to the basal group. It seems that nociceptive stimulation induced the maximum release of corticosterone, so that when animals were also subjected to threatening situations (e.g., sEPM and oEPM), the levels of corticosterone remained unchanged. Thus, it is possible that pain stimulation produced a maximal physiological response on corticosterone levels of this species to an aversive experience. This conclusion is strengthened when we observe the corticosterone levels of eEPM-exposed mice. This group showed higher corticosterone levels than eEPM-exposed mice without the formalin injection. Moreover, eEPM-exposed mice that received the nociceptive stimulation displayed a similar corticosterone response profile to that observed for sEPM- and oEPM-exposed mice. In other words, all three groups that had received the formalin injection showed similar increases in corticosterone levels.

It is important to stress that sEPM- and oEPM-exposed mice that had not received the nociceptive stimulus exhibited higher secretion of corticosterone than equivalent eEPM mice, confirming the aversiveness of the mazes with open arms. Unexpectedly, sEPM-exposed mice exhibited similar levels of corticosterone compared to oEPM-exposed animals, suggesting that the experience of mice to a totally open EPM would not be more aversive than the exposure to a maze with protected closed arms. However, it is likely that the exposure to the sEPM has caused a maximal response on corticosterone levels. In this context, it has been shown that circulating corticosteroids reach a peak within minutes of exposure to aversive events (Palkovits, 1987). Moreover, there are regulatory mechanisms which maintain these levels within an appropriate interval as a form of prevention against deleterious effects to the organism (Munck

et al., 1984). One example of these mechanisms is the regulation of the HPA axis activity by glucocorticoids; it means that this component may inhibit its own release (Akil and Morano, 1995).

Present results also showed that even exposure to the control situation (eEPM) caused an increase in the plasma levels of corticosterone. A possible explanation for these high levels in the eEPM-exposed group may be that the mice had not been familiarized with that place. It is likely that this response was due to the novelty that mice were exposed to. This conclusion has been raised in previous findings (Pellow et al., 1985) that demonstrated that a simple exposure to a novel place, such as the enclosed arm of the sEPM, increases plasma corticosterone release in rats.

The present study confirmed previous findings (Cornélio and Nunes-de-Souza, 2009; Mendes-Gomes and Nunes-de-Souza, 2005, 2009; Mendes-Gomes et al., 2011) demonstrating that only exposure to the oEPM (but not to the sEPM) induced highly significant antinociception (Table 1). Considering the present results did not show differences in the plasma levels of corticosterone in sEPM- and oEPM-exposed mice, we suggest that the release of corticosterone is not crucially involved in the mediation of oEPM-induced antinociception.

Supporting this hypothesis, present results also showed that surgical removal of adrenal glands did not disrupt the oEPM-induced antinociception. Actually, ADX did not alter nociceptive response observed even in the first phase of the formalin test. In this context, we have recently demonstrated that ADX changed neither the first phase of the formalin test nor oEPM-induced antinociception in rats (Cornélio and Nunes-de-Souza, 2009). Whereas previous findings have corroborated the inability of ADX to alter the nociceptive response to the formalin test (e.g., Taylor et al., 1998), contrasting evidence has emphasized that both phases of nociception response induced by formalin are decreased in ADX rats (Vissers et al., 2004). Moreover, Yarushkina (2008) has demonstrated that glucocorticoids can mediate stress-induced analgesia.

Regarding the anxiety indices and locomotor activity observed in sEPM-exposed mice, with or without prior formalin injection, present results showed that nociceptive stimulation did not increase the avoidance of the open arms. In other words, a similar pattern of maze exploration was observed in mice subjected or not to the nociceptive test, suggesting that pain per se does not affect significantly the conventional measures of anxiety (% open arm entries and % open arm time) in the sEPM. In this context, it has been demonstrated that although exogenous corticosterone administration increased (Calvo et al., 1998) or decreased (Andreatini and Leite, 1994; McBlane and Handley, 1994) anxiety-like behavior in the sEPM, administration of metirapone, a corticosterone synthesis inhibitor, did not change open arm avoidance (Calvo et al., 1998; Rodgers et al., 1999), suggesting that endogenous corticosterone may not be directly involved in mediation of the emotional responses in the EPM (Calvo et al., 1998). In this context, the present results also show that while the formalin injection increased the corticosterone response in all EPM-exposed animals, this effect did not lead to a reduction in the open arm exploration in sEPM-exposed mice. These apparently inconsistent results remain to be clarified in further studies.

The behavioral profile of mice exposed to the three types of EPM, with or without prior formalin injection, was also investigated. As shown in Table 2, sEPM- and oEPM-exposed mice displayed lower numbers of arm entries than eEPM-exposed mice. In addition, sEPM-exposed mice spent more time on the center platform. These results corroborate previous studies (Mendes-Gomes and Nunes-de-Souza, 2005, 2009; Mendes-Gomes et al., 2011) suggesting reduced locomotion in situations where a potentially aversive stimulus is present (i.e., the open arms). A similar behavioral pattern was observed in eEPM- and oEPM-exposed mice with formalin injection after adrenal removal (Table 3). Although the total arm entries of oEPM-exposed mice did not differ from eEPM-exposed mice in ADX animals, the former group spent more time on the center platform, emphasizing the aversiveness of the

open arms. Finally, it is also important to point out that neither nociceptive stimulation nor ADX surgery produced substantial changes in locomotion or in time spent on the central square in mice exposed to any of the three types of EPM.

In conclusion, the present study suggests that corticosterone does not exert a crucial role in the modulation of oEPM-induced antinociception. In addition, the lack of anxiolytic-like effects observed in earlier work in which mice subjected to the nociceptive test and injected with midazolam into the PAG (Mendes-Gomes and Nunes-de-Souza, 2005) or had this midbrain structure bilaterally lesioned (Mendes-Gomes and Nunes-de-Souza, 2009) is unlikely to be due to the release of this glucocorticoid.

## Acknowledgments

We thank Elizabete Zocal Paro Lepera and Rosana Finoti Pupim Silva for their excellent technical assistance. This study was supported by FAPESP, CNPq and PADC/FCF-UNESP. J. Mendes-Gomes and T.T. Miguel were supported by FAPESP fellowship (Proc. 05/01988-3 and 05/05171-1), V.C.S. Amaral by CNPq fellowship (Proc.142266/2008-6) and R.L. Nunes-de-Souza received a CNPq research fellowship (Proc. 303580/2009-7).

## References

- Abbott, F.V., Franklin, K.B.J., Westbrook, R.F., 1995. The formalin test: scoring properties of the first and second phases of the pain response in rats. *Pain* 60, 91–102.
- Akil, H.A., Morano, M.I., 1995. Stress. In: Bloom, F.E., Kupfer, D.J. (Eds.), *Psychopharmacology: The Fourth Generation of Progress*. Raven Press, New York, pp. 773–785.
- Amaral, V.C., Santos Gomes, K., Nunes-de-Souza, R.L., 2010. Increased corticosterone levels in mice subjected to the rat exposure test. *Horm. Behav.* 57, 128–133.
- Andreatini, R., Leite, J.R., 1994. The effect of corticosterone in rats submitted to the elevated plus-maze and pentylenetetrazol-induced convulsions. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 18, 1333–1347.
- Bon, K., Wilson, S.G., Mogil, J.S., Roberts, W.J., 2002. Genetic evidence for the correlation of deep dorsal horn Fos protein immunoreactivity with tonic formalin pain behavior. *J. Pain* 3, 181–189.
- Calvo, N., Martijena, I.D., Molina, V.A., Volosin, M., 1998. Metyrapone pretreatment prevents the behavioral and neurochemical sequelae induced by stress. *Brain Res.* 800, 227–235.
- Carobrez, A.P., Bertoglio, L.J., 2005. Ethological and temporal analyses of anxiety-like behavior: the elevated plus-maze model 20 years on. *Neurosci. Biobehav. Rev.* 29, 1193–1205.
- Carrasquillo, Y., Gereau, R.W., 2007. Activation of the extracellular signal-regulated kinase in the amygdala modulates pain perception. *J. Neurosci.* 27, 1543–1551.
- Cornélio, A.M., Nunes-de-Souza, R.L., 2009. Open elevated plus maze-induced antinociception in rats: a non-opioid type of pain inhibition? *Physiol. Behav.* 96, 440–447.
- Dubuisson, D., Dennis, S.G., 1977. The formalin test: a quantitative study of the analgesic effects of morphine, meperidine, and brainstem stimulation in rats and cats. *Pain* 4, 161–174.
- Graeff, F.G., Viana, M.B., Tomaz, C., 1993. The elevated T-maze, a new experimental model of anxiety and memory: effect of diazepam. *Braz. J. Med. Biol. Res.* 26, 67–70.
- Handley, S.L., Mithani, S., 1984. Effects of alpha-adrenoceptor agonists and antagonists in a maze-exploration model of fear-motivated behavior. *Naunyn-Schmiedeberg Arch. Pharmacol.* 327, 1–5.
- Herman, J.P., Ostrander, M.M., Mueller, M.K., Figueiredo, H., 2005. Limbic system mechanisms of stress regulation: hypothalamo-pituitary-adrenocortical axis. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 29, 1201–1213.
- Ji, G., Fu, Y., Ruppert, K.A., Neugebauer, V., 2007. Pain-related anxiety like behaviour requires CRF I receptors in the amygdala. *Mol. Pain* 3, 1–5.
- Korte, S.M., 2001. Corticosteroids in relation to fear, anxiety and psychopathology. *Neurosci. Biobehav. Rev.* 25, 117–142.
- Lariviere, W.R., Melzack, R., 2000. The role of corticotropin-releasing factor in pain and analgesia. *Pain* 84, 1–12.
- Le Bars, D., Gozariu, M., Cadden, S.W., 2001. Animal models of nociception. *Pharmacol. Rev.* 53, 597–652.
- Lee, C., Rodgers, R.J., 1990. Antinociceptive effects of plus-maze exposure: influence of opiate receptor manipulations. *Psychopharmacology (Berl)* 102, 507–513.
- Levine, S., 2005. Developmental determinants of sensitivity and resistance to stress. *Psychoneuroendocrinology* 30, 939–946.
- Lister, R.G., 1987. The use of a plus-maze to measure anxiety in the mouse. *Psychopharmacology (Berl)* 92, 180–185.
- Lumb, B.M., 2004. Hypothalamic and midbrain circuitry that distinguishes between escapable and inescapable pain. *News Physiol. Sci.* 19, 22–26.
- Marin, M.T., Cruz, F.C., Planeta, C.S., 2007. Chronic restraint or variable stresses differently affect the behavior, corticosterone secretion and body weight in rats. *Physiol. Behav.* 90, 29–35.
- McBlane, J.W., Handley, S.L., 1994. Effects of two stressors on behaviour in the elevated. *Psychopharmacology (Berl)* 116, 173–182.
- McCall, W.D., Tanner, K.D., Levine, J.D., 1996. Formalin induces biphasic activity in C-fibers in the rat. *Neurosci. Lett.* 208, 45–48.
- Mendes-Gomes, J., Nunes-de-Souza, R.L., 2005. Concurrent nociceptive stimulation impairs the anxiolytic effect of midazolam injected into the periaqueductal gray in mice. *Brain Res.* 1047, 97–104.
- Mendes-Gomes, J., Nunes-de-Souza, R.L., 2009. Anxiolytic-like effects produced by bilateral lesion of the periaqueductal gray in mice: influence of concurrent nociceptive stimulation. *Behav. Brain Res.* 203, 180–187.
- Mendes-Gomes, J., Amaral, V.C., Nunes-de-Souza, R.L., 2011. Ventrolateral periaqueductal gray lesion attenuates nociception but does not change anxiety-like indices or fear-induced antinociception in mice. *Behav. Brain Res.* 219, 248–253.
- Munck, A., Guyre, P.M., Holbrook, N.J., 1984. Physiological functions of glucocorticoids in stress and their relation to pharmacological actions. *Endocr. Rev.* 5, 25–55.
- Neugebauer, V., Li, W., Bird, G.C., Han, J.S., 2004. The amygdala and persistent pain. *Neuroscientist* 10, 221–234.
- Palkovits, M., 1987. Organization of the stress response at the anatomical level. *Prog. Brain Res.* 72, 47–55.
- Pellow, S., Chopin, P., File, S.E., Briley, M., 1985. Validation of open: closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. *J. Neurosci. Methods* 14, 149–167.
- Pinheiro, S.H., Zangrossi Jr., H., Del-Ben, C.M., Graeff, F.G., 2007. Elevated mazes as animal models of anxiety: effects of serotonergic agents. *An Acad. Bras. Cienc.* 79, 71–85.
- Rodgers, R.J., Johnson, N.J.T., 1995. Factor analysis of spatiotemporal and ethological measures in the murine elevated plus-maze test of anxiety. *Pharmacol. Biochem. Behav.* 52, 297–303.
- Rodgers, R.J., Haller, J., Holmes, A., Halasz, J., Walton, T.J., Brain, P.F., 1999. Corticosterone response to the plus-maze: high correlation with risk assessment in rats and mice. *Physiol. Behav.* 68, 47–53.
- Russo, A.S., Guimarães, F.S., De Aguiar, J.C., Graeff, F.G., 1993. Role of benzodiazepine receptors located in the dorsal periaqueductal grey of rats in anxiety. *Psychopharmacology (Berl)* 110, 198–202.
- Sarnyai, Z., Biro, E., Penke, B., Telegdy, G., 1992. The cocaine-induced elevation of plasma corticosterone is mediated by endogenous corticotropin-releasing factor (CRF) in rats. *Brain Res.* 589, 154–156.
- Steimer, T., Driscoll, P., 2003. Divergent stress responses and coping styles in psychogenetically selected Roman high-(RHA) and low-(RLA) avoidance rats: behavioural, neuroendocrine and developmental aspects. *Stress* 6, 87–100.
- Sufka, K.J., Watson, G.S., Nothdurft, R.E., Mogil, J.S., 1998. Scoring the mouse formalin test: validation study. *Eur. J. Pain* 2, 351–358.
- Tanimoto, S., Nakagawa, T., Yamauchi, Y., Minami, M., Satoh, M., 2003. Differential contributions of the basolateral and central nuclei of the amygdala in the negative affective component of chemical somatic and visceral pains in rats. *Eur. J. Neurosci.* 18, 2343–2350.
- Taylor, B.K., Akana, S.F., Peterson, M.A., Dallman, M.F., Basbaum, A.I., 1998. Pituitary-adrenocortical responses to persistent noxious stimuli in the awake rat: endogenous corticosterone does not reduce nociception in the formalin test. *Endocrinology* 139, 2407–2413.
- Tjolsen, A., Berge, O.G., Hunskaar, S., Rosland, J.H., Hole, K., 1992. The formalin test: an evaluation of the method. *Pain* 51, 5–17.
- Vissers, K.C., De Jongh, R.F., Crui, B.J., Vinken, P., Meert, T.F., 2004. Adrenalectomy affects pain behavior of rats after formalin injection. *Life Sci.* 74, 1243–1251.
- Yarushkina, N.I., 2008. The role of hypothalamo-hypophyseal-adrenocortical system hormones in controlling pain sensitivity. *Neurosci. Behav. Physiol.* 38, 759–766.
- Yokoro, C.M., Tatsuo, M.A., Pereira, L.S., Alves, D.L., Francischi, J.N., 2003. Role of endogenous glucocorticoids in hyperalgesia and edema in old arthritic rats. *Braz. J. Med. Biol. Res.* 36, 77–83.