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Previous stress exposure enhances both anxiety-like behaviour and p35 levels in the basolateral amygdala complex: Modulation by midazolam

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Abstract

Stress exposure induces long lasting neurobiological changes in selected brain areas, which could be associated with the emergence of negative emotional responses. In the present study, previously restrained animals exhibited excessive anxiety one day later in the elevated plus maze. We explore whether stress exposure affects the expression levels of cyclin-dependent kinase 5 (Cdk5) and of its activator protein p35, in diverse amygdaloid nuclei. Stress exposure enhanced p35 levels selectively in the basolateral amygdala (BLA). This up-regulation might be functionally associated with the occurrence of exaggerated anxiety since such emotional response was selectively reversed by an intra-BLA infusion of olomoucine, a Cdk5 inhibitor, 15 min prior to the restraint session. Moreover, pre-treatment with midazolam, a benzodiazepine ligand, not only prevented the excessive anxiety but also attenuated the p35 increase in the BLA of stressed rats. In conclusion, we suggest a pivotal role of the Cdk5/p35 complex, specifically in BLA in the excessive anxiety induced by a previous stressful experience.
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1. Introduction

An emerging body of literature has revealed that stressful experiences often result in long-lasting inappropriate anxiety and/or excessive fear in subsequent exposure to mildly aversive or neutral stimuli (Martijena et al., 1997, 2002; McGaugh and Roozendaal, 2002; Korte and De Boer, 2003; Rodriguez Manzanares et al., 2005; Calfa et al., 2006, 2007). These disturbed behavioural responses have been reported using diverse experimental paradigms and following the exposure to a variety of stressful stimuli (Korte and De Boer, 2003; Adamec et al., 2005; Calfa et al., 2006, 2007). This process has been tentatively defined as stress or emotional sensitization (Stam et al., 2000; Wiedenmayer, 2004).

The amygdaloid complex is a key component in the neural circuitry that coordinates negative emotional responses to threatening stimuli (LeDoux, 1994; Herman and Cullinan, 1997; Adamec et al., 1999; Davis, 2002). It also plays a pivotal role in mediating fear associative learning (Fendt and Fanselow, 1999;

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LeDoux, 2000). This brain region attributes affective significance to relevant environmental information and conveys it to areas involved in the promotion of emotional and behavioural responses (Aggleton, 1993). Moreover, this complex receives sensory information from all modalities at all levels (Sah et al., 2003). Briefly, the basolateral amygdaloid complex (BLA; consisting of the lateral, basal and accessory basal nuclei) is the primary input of this brain area, which receives cortical and subcortical projections. This information is relayed, within an intra-amygdaloid circuitry, to the central nucleus (CeA), which forms the major output area of the amygdaloid complex. In fact, the CeA controls the expression of the emotional response, including a distinctive pattern of behavioural, autonomic and neuroendocrine reactions to stressful stimuli, via projections principally to the midbrain and brainstem nuclei (Davis, 1992; Sawchenko et al., 1996; LeDoux, 2000; Petrovich and Swanson, 1997). Several reports suggest that environmental information can be processed by mechanisms intrinsic to amygdala networks in order to integrate sensory inputs and generate appropriate emotional responses according to changing environmental demands.

Finally, this brain structure also plays a pivotal role in the influence of stress hormones on emotional responses. For instance, compelling evidence indicates that glucocorticoids locally infused into the amygdala modulate the formation of emotional memory (Roozendaal and McGaugh, 1997; Sandi et al., 1997; McGaugh and Roozendaal, 2002).

Despite the overwhelming behavioural evidence supporting the role of the amygdala mediating stress-related emotional responses such as anxiety and fear, the underlying molecular events in stress sensitization in this particular brain area are not currently established. Recently, we described that the exposure to a restraint event selectively enhanced both the expression and activity of cyclin-dependent kinase 5 (Cdk5) in the lateral septum (Bignante et al., 2008), a brain area identified as an important neuroanatomical locus in the modulation of the behavioural outcome and coping strategies to stressful stimuli. In addition, this brain area has been indicated as an important site of action for anti-anxiety drugs (Gray and McNaughton, 2000).

The essential role of Cdk5 and its activators, p35 and p39, in neuronal processes for normal brain development has been well established (Nikolic et al., 1996; Ohshima et al., 1996; Paglini et al., 1998; Chae et al., 1997; Paglini and Cáceres, 2001; Dhavan and Tsai, 2001). Besides, enhanced Cdk5/p35 activity has been associated with the formation of fear memory (Fischer et al., 2002). Coincidently, this enzyme was suggested to play a key role in the generation of synaptic plasticity thought to be required for memory formation (Fischer et al., 2002, 2005; Hawasli and Bibb 2007). What is more, pharmacological blockade of septal Cdk5 prevented associative learning (Fischer et al., 2002).

Given that amygdala has a pivotal role in the generation of fear memory and for processing threatening environmental information, the principal goal of the present study was to examine whether stress exposure affects the expression of Cdk5 and of its activator p35, in diverse amygdaloid nuclei. In order to analyze the role of these proteins in the stress-induced sensitization process, animals were locally infused with olomoucine, an inhibitor of Cdk5 activity (Veselý et al., 1994; Bibb et al., 2001). This was performed prior to stress exposure, either into the BLA or CeA and the next day the rats were tested in the elevated plus maze (EPM), a valid animal model of anxiety (Pellow et al., 1985; Cruz et al., 1994). Next, we investigated the influence of midazolam (MDZ), a benzodiazepine ligand, prior to stress exposure, on the potential stress-induced changes on anxiety-like behaviour. Finally, we evaluated the influence of MDZ on Cdk5 and p35 expression in several nuclei of the amygdaloid complex of stressed animals.

2. Experimental procedures

2.1. Animals

Adult male Wistar rats (65–75 days), bred in our colony and weighing 280–320 g were housed in groups of 2–3 per cage with food and water ad libitum. They were maintained in a 12 h light–dark cycle (lights on at 07:00 a.m.) at a constant room temperature of 21–22 °C. Rats were handled during the week before the experimental procedure, in order to habituate them to manipulation. This habituation consisted in the transportation of the animals to an experimental room, removing them from their cages, the handling of each animal during 1 min and returning them to their home cages. This procedure was repeated twice a day during four consecutive days before the experiments.

All the experiments were performed during the light cycle between 10:00 a.m. and 03:00 p.m.). Procedures were conducted in accordance with the National Institutes Health Guide for the Care and Use of Laboratory Animals, as approved by the Animal Care and Use Committee of the Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, and by National Department of Animal Care and Health (SENASA – ARGENTINA). Efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2. Stressor

Rats were transferred in their home cages to an experimental room, and placed inside a plastic cylindrical restrainer fitted close to the body for 30 min. This restrainer contained numerous holes to allow normal respiration of the animal which had only the tip of its nose and tail free (Cancela et al., 1998). At the end of the stress session, restrained rats were returned to their home cages, and finally to their colony room. This procedure was selected based on prior findings from our laboratory using a similar stress protocol to that performed in the present study (Martijena et al., 1997, 2002, Rodriguez Manzanares et al., 2005, Isoardi et al., 2007). These studies showed that this stressful situation attenuated the inhibitory GABAergic control in BLA, resulting in neuronal hyperexcitability and facilitated the induction of LTP in BLA, associated with the enhancement of fear memory. Control rats were also transferred to the experimental room, handled for a minute, and then returned to the colony room.

2.3. Immunohistochemical analysis

The procedure used was similar to one previously described (Bignante et al., 2008). Briefly, rats were deeply anesthetized with chloral hydrate (400 mg/kg i.p.) and perfused transcardially with saline followed by a solution of 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS), pH 7.4. Brains were removed and post-fixed in the same fixative overnight at 4 °C. They were then placed in 30% sucrose in PBS for 72 h, and coronally sectioned in a cryostat into 30 μm thick coronal slices (Leica, Nussloch, Germany). Free floating sections were incubated for an hour in a solution of 3% hydrogen peroxide and 10% methanol to eliminate peroxidase activity. This was followed by a blocking solution (5% bovine-serum albumin (BSA) and 0.3% Tritón X-100 in 0.1 M PBS) and finally by a solution containing rabbit polyclonal Cdk5 (C-8) or p35 (C-19) antibodies (Santa

Cruz Biotechnology, Santa Cruz, CA, USA; catalogue number: sc-173 and sc-820 respectively, see Paglini et al., 1998), 1:400 in 0.1 M PBS containing 1% BSA and 0.1% Tritón X-100 at 4 °C. Forty-eight hours later, sections were incubated with biotinylated mouse secondary anti-rabbit antibody (Vector Laboratories, Burlingame, CA, USA), diluted 1:200 in 0.1 M PBS containing 1% BSA, followed by an avidin–biotin–peroxidase (ABC) complex (Vector ABC kit, Vector Laboratories, Burlingame, CA) for 1 h at room temperature. For visualization, 3′-diamino-benzidine tetrahydrochloride (DAB Sigma) was used as a chromogen (Sigma fast tablet set) (0.05% of DAB and 0.0006% of hydrogen peroxide solution). The coloration reaction was stopped after 5 min by adding PBS. Brain sections were mounted onto glass slices, dehydrated and coverslipped, prior to viewing with light microscopy.

2.4. Quantification of labeled cells

Positive Cdk5 or p35 cells were labeled with a brown coloration. Slices were viewed and photographed, using light field microscopy (Zeis Axioplan) with Metamorph computer software at a magnification of 200X. Positive cells were counted using a computational software (a SCION program from the NIH). This quantification was carried out by a person not involved in the experiment, and performed using an identical size area (0.16 mm2) of the same shape. The anteroposterior (AP) coordinates from bregma of the basolateral amygala (BLA), which were included for detailed analysis, were AP: −2.30 to −3.30; and for the central amygdala (CeA) were AP: −1.80 to −2.80, according to the Paxinos and Watson atlas (Paxinos and Watson, 1997). Three brain sections per rat were used to quantify the number of cdk5- or p35-immunoreactive cells in each area.

2.5. Elevated plus-maze

The apparatus consisted of a maze made in black acrylic, elevated 50 cm above the floor with two opposite open arms (50×10 cm) and two opposite closed arms (50×10×40 cm) disposed like a plus sign with a central square of 10 cm^2. Each test lasted 5 min and was performed in a quiet and dimly illuminated room (4 lx). Each rat was placed in the central square of the maze facing one closed arm. The scores analyzed were the time spent in open and closed arms, and the number of entries to both arms. These data were calculated as the percentage of time spent on open arms which was used as an anxiety index (time spent on open arms relative to the time spent on open and closed arms). The number of entries to the closed arms was also assessed, as indicative of exploratory activity (Cruz et al., 1994; Rodgers and Cole, 1994; Martijena et al., 2002). Behavioral assessment was carried out by a person who was blind to the experimental condition of the each animal.

2.6. Implantation of guide cannulas for intracerebral infusions

Animals were anesthetized with an intraperitoneal injection of ketamine (55 mg/kg) — xylazine (11 mg/kg) and placed on a stereotaxic instrument (Stoelting Inc., Wood Dale, IL) with the incisor bar set at −3.3 mm. The surgery consisted of the implantation of bilateral guide cannulas (22-gauge stainless steel tubing of 12 mm length) into the BLA or CeA using the following coordinates: AP, −2.4 mm; lateral, ±5 mm; DV, −6.2 mm; and AP, −2.1 mm; lateral, ±4 mm; DV, −5.7 mm; respectively, from the skull according to the Paxinos and Watson atlas (1997). These coordinates were established from pilot studies in our laboratory. The guide cannulae were secured in place using acrylic cement and two stainless steel screws anchored to the skull. Stainless dummy cannulas protruding 0.5 mm beyond the tips were placed inside the guide cannulas to prevent occlusion. After surgery, animals received a subcutaneous injection of a penicillin/ streptomycin suspension to prevent infections. Rats were handled every day during the week before the beginning of the experiments, and familiarized with the infusion procedure in order to minimize non-specific stress responses.

2.7. Intracerebral infusions

Microinfusions were made using 33-gauge infusion cannulae that extended 2 mm beyond the guide cannulae implanted in the BLA or CeA. The infusion cannulae were connected via polyethylene tubing (PE 10, Becton Dickinson, MD) to a 10 µl microsyringe (Hamilton, Reno, NV) mounted on a microinfusion pump (Harvard Apparatus, Holliston, MA). Each rat was bilaterally administered with 0.5 µl/side at a flow rate of 0.5 µl/min over a period of 60 sec. After completion of the volume injection, the infusion cannulae were kept in place for an additional period of 30 s to allow diffusion of the drug.

2.8. Drugs

A solution of olomoucine (Calbiochem, San Diego, CA) dissolved in DMSO was diluted with PBS to a final concentration of 20 ng/0.5 µl for intracerebral infusions. This dose was selected based on previous reports which showed a selective inhibition of Cdk5 following local infusion of this drug (Bibb et al., 2001). Moreover, these authors found a similar effect between roscovitine and this dose of olomoucine after intracerebral infusions. Olomoucine acts by competing for the ATP binding domain of the kinase (Veselý et al., 1994). Control rats were infused with 0.5 µl of vehicle (DMSO 40% in PBS). Midazolam (Fada Pharma, Buenos Aires, Argentina) was dissolved with distilled water to achieve a concentration of 0.5 mg/ml for i.p. administration. Control rats were injected with saline.

2.9. Histology

At the end of the experiment, cannulated rats were sacrificed with an overdose of chloral hydrate, decapitated, and their brains removed. The brains were fixed in a formalin solution and sectioned in a cryostat (Leica, Nussloch, Germany) for the localization of the injection sites, with the extent of tissue damage being examined under a light microscope. Only those animals with adequate injection sites were considered for statistical analysis.

2.10. Statistical analysis

The data collected were processed using the software Statistic 6.0, values representing the mean ± S.E.M were calculated. The data were analyzed using a Student's *t*-test or a two way ANOVA. In the last case, ANOVAs were followed by post hoc analysis (Newman-Keuls analysis) to enable specific group comparisons. $p<0.05$ was considered to be significant.

3. Results

3.1. Experiment 1. A single restraint session selectively increased the p35 levels in BLA

The aim of this experiment was to explore whether a single restraint session modifies the levels of Cdk5 and/or p35 in amygdalar nuclei. Rats were assigned to the following experimental conditions: restrained for 30 min (RES group) or without aversive stimulation (CON group). These animals were intracardially perfused and their brains extracted for immunohistochemical analysis. The RES group was sacrificed 30 min after restraint. This time point was selected based on previous data showing maximal Cdk5 expression and activity at 30 min following stress exposure (Fischer et al., 2002).

Levels of Cdk5 expression were not modified by restraint in any nuclei of amygdalar complex. We observed a trend for Cdk5 levels in BLA to increase in restraint group relative to control group although this increase was not statistically significant ($t=-2.0027$ $p=0.0589$). However, stressed rats showed a selective increase in p35 levels in BLA respect to control rats (Fig. 1).

A Student test revealed a significant effect of stress on the number of p35 positive cells in BLA as a consequence of stress exposure ($t=-12.6027$ $p<0.0001$). In contrast, this effect was absent in any other nuclei of the amygdalar complex: CeA ($t=-1.5526$ $p=0.1428$), LA ($t=0.3360$ $p=0.7405$), MeA ($t=-0.3225$ $p=0.7503$) (Table 1).

Taking into account that the binding of p35 is sufficient to activate Cdk5 (Lew et al., 1994,Tsai et al., 1994; Dhavan and Tsai, 2001; Wei et al., 2005) and that in vivo evidences have demonstrated that the levels of p35 is considered a rate-limiting factor for the up-regulation of Cdk5 activity, despite the fact that the expression levels of Cdk5 remain invariable (Takahashi et al., 2005); the increased expression of p35 reported in this experiment implies an enhanced activity of this kinase complex Cdk5/p35.

3.2. Experiment 2. Intra-BLA infusion of olomoucine, but not intra-CeA, prevented the emergence of excessive anxiety induced by stress

To investigate the functional role of Cdk5 in the BLA or CeA on the emotional sensitization process after stress exposure, we performed blocking experiments with a local infusion of olomoucine, a Cdk5 inhibitor. Rats were cannulated in the BLA or CeA and distributed randomly into four groups: OLO CON (animals infused with olomoucine without restraint); OLO RES (rats infused with olomoucine and restrained for 30 min); VEH CON (rats infused with vehicle without restraint) and VEH RES (rats infused with vehicle and restrained for 30 min). Stressed rats were either infused with olomoucine or vehicle 15 min before stress exposure. All subjects were tested in the EPM 24 h after the end of the restraint experience.

Fig. 2 shows the injection site of animals cannulated in BLA or CeA. As expected, rats infused with vehicle and subjected to restraint (group VEH RES) exhibited an increased suppression of open-arm exploration in the EPM, indicative of excessive anxiety. In contrast, the behavior in the EPM of animals that were infused with olomoucine intra-BLA previous to stress was similar to that exhibited by unstressed rats (Fig. 3). However, olomoucine intra-CeA failed to prevent the occurrence of exaggerated anxiety following stress exposure (Fig. 4).

For rats infused intra-BLA, a two-way ANOVA showed a significant effect of treatment (CON or RES) ($F(1,30)=9.5591$ $p<0.005$), of pretreatment (VEH or OLO) ($F(1,30)=8.9273$ $p<0.01$), and of pretreatment X treatment interaction ($F(1,30)=14.0949$ $p<0.001$) on the percentage of time spent in open arms. Furthermore, the Newman-Keuls post hoc test revealed a reduction in the percentage of time spent in open arms following restraint, with this effect being fully prevented by prior administration with intra-BLA olomoucine ($p<0.001$).

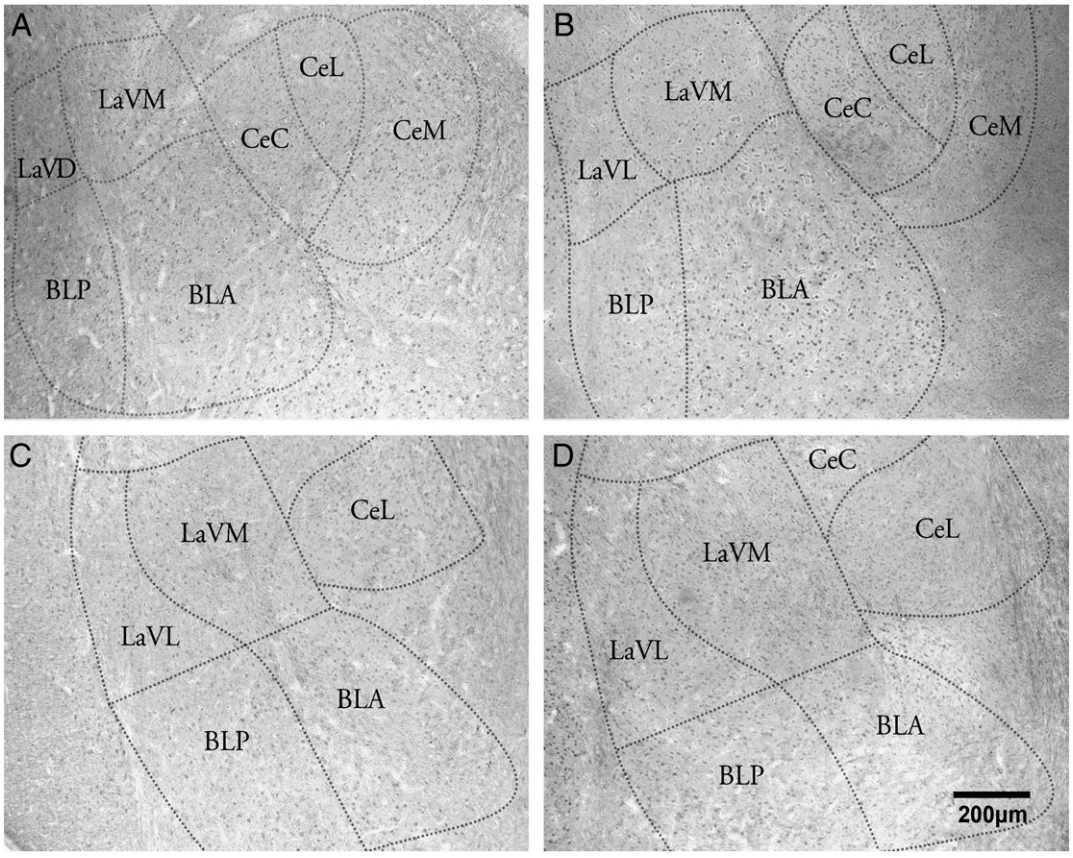

Figure 1 Representative photomicrographs of p35 immunoreactivity (100×). P35 immunoreactivity in basolateral and central nuclei of the amygdala from A) control rats, B) restrained rats, C) unstressed rats injected with MDZ i.p. and D) restrained rats with previous MDZ administration i.p. (DAB stain).

Table 1 Number of p35 and Cdk5 positive cells in different nuclei of amygdala followings stress exposure. Quantification of p35 and Cdk5 positive cells in basolateral (BLA), central (CeA), lateral (LA) and medial (MeA) amygdala. The values represent number of positive cells stained with DAB in an area of 0.16 mm^2 (mean ± SEM) (*) $p<0.0001$ vs. control group ($n=4$).

Amigdalar Nuclei	p35 positive cells CON	p35 positive cells RES	Cdk5 positive cells CON	Cdk5 positive cells RES
BLA	325 ± 11	**495 ± 9** (*)	390 ± 13	466 ± 15
CeA	644 ± 29	714 ± 36	560 ± 30	614 ± 26
LA	216 ± 21	207 ± 14	202 ± 15	215 ± 21
MeA	307 ± 18	316 ± 24	271 ± 30	290 ± 14

No significant effect on the number of closed arms entries was detected ($F(1,30)=0.2252$ $p=0.6385$), suggesting that the potentiation of anxiety-like behavior induced by restraint is not due to a reduction in exploratory activity (Cruz et al., 1994) (Data not shown).

For animals infused intra-CeA, the ANOVA revealed a significant effect of treatment (CON o RES) ($F(1,31)=27{,}3879$ $p<0.0001$), but no effect of pretreatment (VEH o OLO) ($F(1,31)=0.16438$ $p=0.6879$), or of pretreatment and treatment interaction ($F(1,31)=0.18652$ $p=0.6688$). The post hoc analysis revealed a reduction in the percentage of time spent in the open arms produced by restraint, which was not modified by a previous infusion of olomoucine intra-CeA ($p<0.01$). The exploratory activity was not affected by those treatments, since the number of entries to closed arms was the same in all experimental groups ($F(1,31)=0.9763$ $p=0.3308$).

3.3. Experiment 3. Midazolam pretreatment attenuated both the increase of p35 levels in BLA and the excessive anxiety induced by stress exposure

The first part of this experiment was performed to examine the potential efficacy of a 0.5 mg/kg (i.p.) MDZ dose in preventing the increased anxiety promoted by prior stress exposure. To this end, rats were injected with MDZ or vehicle and 20 min later subjected to restraint for 30 min. One day later, all animals

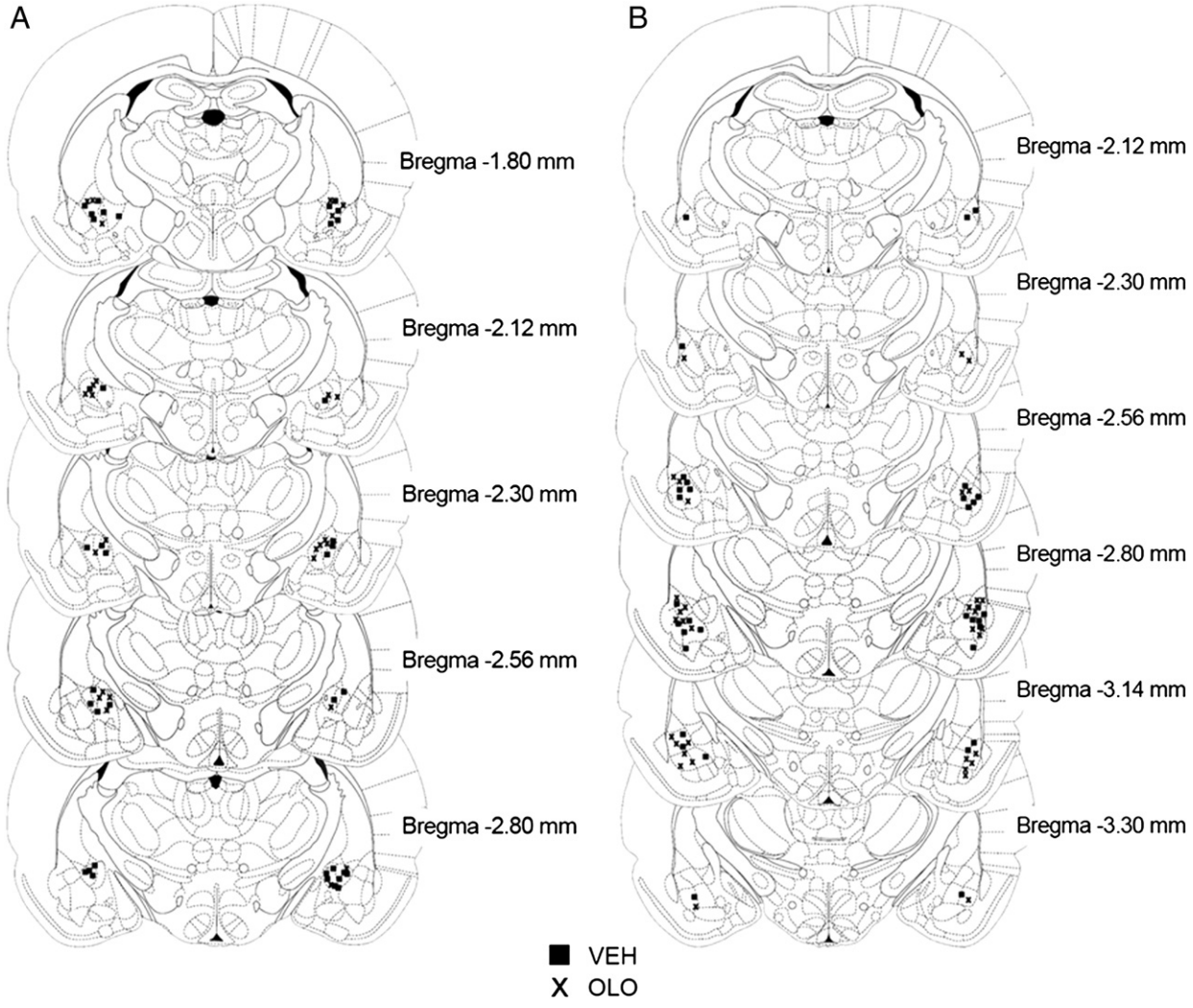

Figure 2 Placement of infusion cannulas. Schematic drawings of coronal sections of the rat brain show the location of the cannulas in the BLA, olomoucine (**x**) and vehicle (■). These drawings were adapted from Paxinos and Watson (1997).

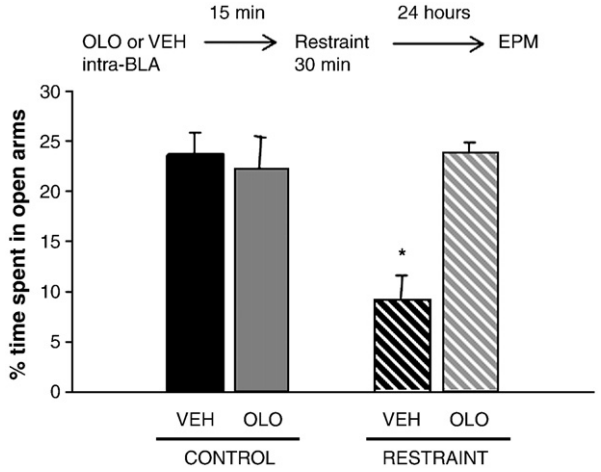

Figure 3 Effect of restraint on the percentage of time spent on open arms in the EPM: influence of previous intra-BLA infusion of vehicle or olomoucine (olo). Restrained rats exhibited a reduction in the percentage of time spent on open arms. This effect was prevented by the previous local infusion of olo into the BLA. Bars represent the means ± S.E.M. of the percentage of time spent in open arms relative to total time spent on all four arms. *$p<0.001$ vs. all the other groups ($n=8–9$).

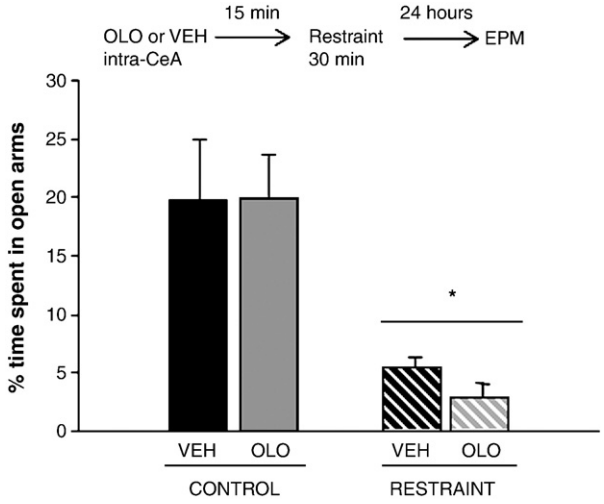

Figure 4 Effect of restraint on the percentage of time spent in open arms of the EPM: influence of previous intra-CeA infusion of vehicle or olomoucine (olo). Restrained rats exhibited a reduction in the percentage of time spent in open arms. This effect was not attenuated by the previous local infusion of olo into the CeA. Bars represent the means ± S.E.M. of percentage of time spent in open arms relative to the total time spent on all four arms. *$p<0.01$ vs. control groups ($n=8–11$).

were tested in the EPM. Rats were distributed randomly into the following groups: MDZ RES (injected with MDZ and restrained), MDZ CON (injected with MDZ without restraint), VEH RES (injected with vehicle and restrained), VEH CON (injected with vehicle without restraint).

In an additional experiment, the levels of p35 in BLA and CeA were assessed by immunohistochemistry for similar experimental groups to those described above. Rats were always sacrificed 30 min after the end of the restraint session.

Animals injected with saline and later subjected to restraint showed an excessive anxiety in the EPM since they exhibited a reduction in the percentage of time spent on open arms. However, when MDZ was administered previous to restraint, animals behaved as control unstressed animals (Fig. 5), indicating that MDZ prevented the emergence of stress-induced excessive anxiety. The number of entries in closed arms was not modified in any experimental groups (Data not shown).

A two-way ANOVA revealed a significant effect of pretreatment (VEH or MDZ) ($F(1,52)=4.6274$ $p<0.05$), of treatment (CON or RES) ($F(1,52)=4.0769$ $p<0.05$), and of interaction between pretreatment and treatment ($F(1,52)=11.6612$ $p<0.005$). The post-hoc Newman-Keuls test revealed a reduction in the percentage of time spent in the open arms produced by restraint, this effect was prevented by a previous MDZ administration ($p<0.01$).

The immunohistochemistry data showed that rats injected with vehicle and later subjected to restraint presented an increase in the number of p35 positive cells in BLA. In contrast, this effect was absent when stressed animals received MDZ prior to stress exposure (Fig. 6).

A two-way ANOVA revealed a significant effect of pretreatment (VEH or MDZ) ($F(1,33)=21.295$ $p<0.0001$), of treatment (CON or RES) ($F(1,33)=36.347$ $p<0.0001$), and of the interaction between pretreatment and treatment ($F(1,33)=41.681$ $p<0.0001$). Additionally, the post-hoc Newman-Keuls analysis revealed an increase in the number of p35 positive cells in stressed rats, this effect was prevented by a previous MDZ administration ($p<0.001$).

Moreover, as depicted in Fig. 7, neither restraint nor MDZ had any effect on the expression of p35 levels in CeA. The ANOVA showed no effect of pretreatment (VEH or MDZ) ($F(1,32)=2.460$ $p=0.1266$), or of treatment (CON or RES) ($F(1,32)=2.436$ $p=0.1284$), or of the interaction between pretreatment and treatment ($F(1,32)=0.385$ $p=0.5392$).

4. Discussion

There is substantial evidence indicating that prior stress exposure facilitates the occurrence of fearful responses and enhances anxiety-like behavior in response to novel environmental stimuli (McGaugh and Roozendaal, 2002; Korte and De Boer, 2003; Rodriguez Manzanares et al., 2005; Calfa et al., 2006, 2007; Vyas et al., 2004). In agreement, our behavioral results showed that animals previously exposed to restraint stress exhibited excessive anxiety in the EPM, as manifested by a significant reduction in open-arm exploration. This finding is consistent with earlier data from this laboratory, using a similar type of stressor to that used in the present study (Martijena et al., 1997, 2002; Bignante et al., 2008). The behavior exhibited in the EPM by stressed animals revealed a clear reduction in the conflict approach/avoidance; in contrast to this behavior pattern, an increased exploration of the open arm is the typical response to anxiolytic drugs (Albrechet-Souza et al., 2007), which has been used as the main reference to validate the EPM as an animal model of anxiety.

At the cellular level, the current immunohistochemical experiments showed that stress exposure resulted in a concomitant increase of p35 positive cells in BLA, but not in other amygdalar nuclei. The fact that such an enhancement

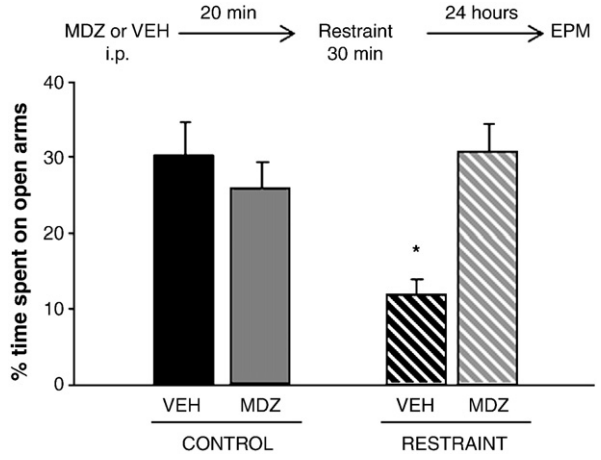

Figure 5 Effect of restraint on the percentage of time spent in open arms in the EPM: influence of previous MDZ administration. Restrained rats showed a reduction in the percentage of time spent in open arms, and this effect was prevented by a previous MDZ i.p. injection. Bars represent the means ± S.E.M. of the percentage of time spent in open arms relative to the total time spent on all four arms. *$p<0.01$ vs. all the other groups ($n=13–15$).

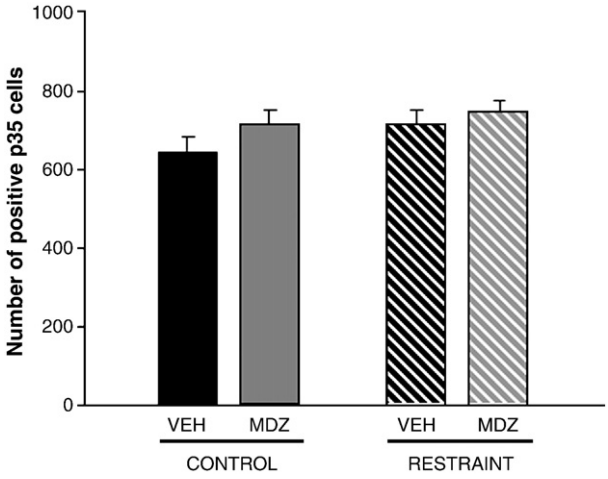

Figure 7 Effect of restraint on the number of p35 positive cells in the CeA of animals previously administered with vehicle or MDZ. Neither restraint nor MDZ altered the levels of p35 positive cells in the CeA. Bars represent the means ± S.E.M. of the number of cells positive for p35 in CeA ($n=4$).

was selectively induced in BLA discard that this effect is due to a non-specific consequence of the aversive experience. Despite that MeA has been suggested to participate in the regulation of the stress-induced response of hypothalamic-pituitary-adrenal axis (Belda et al., 2008), no changes on the levels of Cdk5 and p35 proteins after an acute restraint session were observed in the present study. However, we cannot rule out a role of this area on the stress sensitization process.

It is tempting to propose that a selective up-regulation of BLA p35 could be in fact related to the emergence of a negative emotional response elicited by the threatening stimulus. In line with this argument, our findings also revealed that the excessive anxiety-like behavior exhibited by stressed animals was reversed by a previous infusion of olomoucine (a Cdk5 inhibitor) into the BLA, but not into the adjacent CeA. Furthermore, if the enhancement of p35 levels in the BLA was indeed functionally related to stress-induced potentiation of anxiety-like behavior then, we predicted that a pharmacological intervention that was able to prevent this exaggerated anxiety should also attenuate the increase of p35 positive cells in the BLA following stress exposure. As expected, our results indicated that prior MDZ administration prevented the stress-induced decline in open arm exploration by restoring "normal" levels of anxiety. Consonant with this finding, we have previously demonstrated that MDZ prevents other stress-induced responses, such as excessive fear (Rodriguez Manzanares et al., 2005). Moreover, a comparable BLA p35 expression was observed between unstressed rats and the stressed animals that received MDZ pretreatment, thus indicating that MDZ prior to restraint prevented the increase of p35 expression in BLA. These results support the idea that enhanced BLA p35 levels might be involved in the cascade of events that culminate in a subsequent excessive anxiety.

Finally, the fact that p35 knockout mice did not exhibit increased anxiety following restraint exposure further supports the view of a functional link between stress-induced excessive anxiety and the up-regulation of p35 levels (Bignante et al., 2008).

The selective involvement of BLA neurons in the occurrence of negative emotional responses induced by stress is supported by a large body of evidence. It is widely known that the activation of BLA is essential for emotional regulation (Adamec et al., 1999; Rainnie et al., 2004; Wu et al., 2007, Rodriguez Manzanares et al., 2005; Vyas et al., 2004). In fact, BLA plays a key role in assigning affective value to environmental demands (Cardinal et al., 2002; Davis and Whalen, 2001; Holland and Gallagher, 1999; LeDoux,

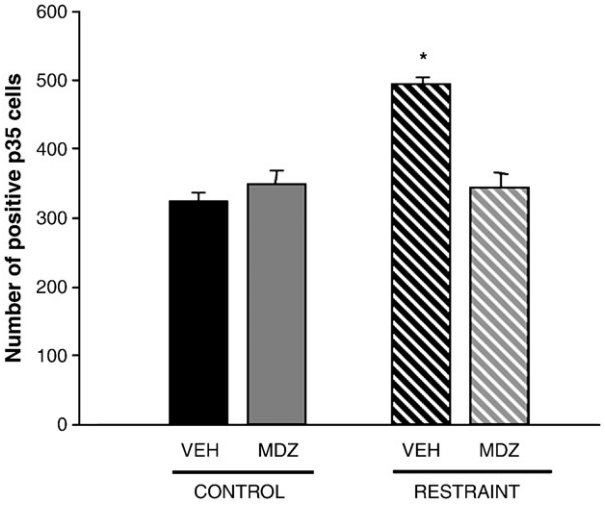

Figure 6 Effect of restraint on the number of p35 positive cells in the BLA of animals previously administered with vehicle or MDZ. Restrained rats exhibited an increase in p35 levels in the BLA. This effect was prevented by a previous MDZ administration. Bars represent the means ± S.E.M. of the number of cells positive for p35 in BLA. *$p<0.001$ vs. all the other groups ($n=4$).

2000). Stress-induced enhancement of BLA neuronal activity has been proposed as a prerequisite for the emergence of excessive anxiety (Rainnie et al., 2004). In addition, the BLA is involved in integrating the stress influence on memory consolidation (see McGaugh, 2000; Roozendaal et al., 2009). The facilitating influence on anxiety-like behavior induced by both environmental threats and stress hormones is accompanied by a selective increase of the dendritic arborization in the BLA (Vyas et al., 2004; Mitra and Sapolsky, 2008), suggesting that the increase in p35 levels as reported in the present study, could have a role on such structural plasticity since Cdk5 activity is related to synaptic remodeling (Fischer et al., 2005; Cheung and Ip, 2007).

Interestingly, corticotropin-releasing hormone (CRH), one of the hormones that has a central role in the processing of stressful stimuli on BLA and on anxiety potentiation, induces an increase in ERK levels selectively in BLA but not in CeA (Refojo et al., 2005). Given that this enzyme is an inductor of p35 transcription (Harada et al., 2001), a selective p35 increase in BLA could be mediated by CRH activation via the ERK cascade. This possibility must be addressed in future studies.

Most of the inputs to the amygdala involve excitatory pathways that use glutamate as a transmitter. The activity of glutamatergic pyramidal projection neurons in the BLA is under the control of a powerful GABAergic inhibitory circuit (Takagi and Yamamoto, 1981; Sah et al., 2003; Dityatev and Bolshakov, 2005). Extensive animal research has sustained the notion that decreased GABAergic neurotransmission in the BLA underlies the emergence of a negative emotional responses such as fear following stress exposure (Rainnie et al., 2004; Rodriguez Manzanares et al., 2005; Van Nobelen and Kokkinidis 2006; Isoardi et al., 2007). Hence, the pharmacological removal of such inhibitory control resulted in an increased anxiety-like behavior (Sanders et al., 1995), enhanced fear memory formation and a facilitated BLA neuroplasticity associated with fear learning (Rodriguez Manzanares et al., 2005). Both effects were reversed by the administration of a positive modulator of GABAa sites.

Collectively, the emergence of excessive fear and anxiety, such as that produced by stress, should be sustained by an increased network excitability mediated by glutamategic neurotransmission in the BLA (Adamec et al., 1999, Rainnie et al., 2004, Rodriguez Manzanares et al., 2005). In fact, if the GABAergic inhibition is reduced, as in the case following stress, then the N-methyl-D-aspartate (NMDA) receptor-mediated excitatory postsynaptic potentials (EPSPs) is unmasked, which leads to enhanced excitability (Rainnie et al., 2004; Isoardi et al., 2007) and to the prolonged activation of glutamatergic receptors, including the NMDA sites. Consonant with this view, Adamec (1999) demonstrated that blocking NMDA sites in BLA prevented the emergence of an emotional sensitized process. Therefore, signaling at NMDA receptors appears to have an important role for the acquisition of aversive experiences. Li and colleagues (2001) reported that Cdk5 phosphorylates the NMDA receptor on its NR2A subunit with this phosphorylation apparently improving the induction of the hippocampal LTP (Li et al., 2001). In line with this argument, we can tentatively suggest that the stress-induced increase of Cdk5 activity in BLA may, in turn, phosphorylate NMDA receptors thus facilitating the occurrence of the plastic process underlying the acquisition of the aversive experience.

Recent reports have suggested that Cdk5/p35 exerts a negative modulatory role on the hippocampus dependent learning (Hawasli et al., 2007; Hawasli and Bibb, 2007). Thus, it is possible to speculate that the activation of Cdk5/p35 complex in BLA or in the hippocampus may result in different behavioral outputs. In agreement, it is known that stress has opposing effects on the hippocampus-dependent behavior compared to those mediated by the amygdala, particularly the BLA. For instance, stressful events were found to impair spatial learning (a hippocampus dependent learning) and LTP generation, and also reduced dentritic branching in the hippocampus. However, in BLA, a similar treatment facilitated the consolidation of emotionally arousing memories, LTP generation and dendritic branching (Vyas et al., 2002, 2004; Roozendaal et al., 2009).

The effect of stress on BLA plasticity may also be due to the conversion of p35 into p25. Classically, the function of p25 has been considered almost exclusively related to neurotoxicity and neurodegeneration (Patrick et al., 1999; Nguyen et al., 2001; Cruz et al., 2003) but there are growing data giving a role for p25 in synaptic plasticity. Fischer and colleagues showed that a transient expression of p25, which did not produce neurodegeneration, could facilitate long-term synaptic changes at the same time as promoting learning and memory (Fischer et al., 2005). Interestingly, the increased activation of NMDA receptors is a crucial step for the conversion of p35 into p25. In fact, increased levels of intracellular Ca^{2+} mediated by NMDA activation is responsible for the activation of calpain, the enzyme that promotes such a conversion (Wei et al., 2005). Given that stress causes an increased excitability of BLA projection neurons, by reducing GABAergic inhibitory currents (Rodriguez Manzanares et al., 2005; Isoardi et al., 2007); the concomitant activation of NMDA sites may facilitate the generation of p25 and the promotion of plastic mechanisms. Future experiments are necessary to evaluate this possibility.

The amygdala, in particular the BLA, is a key component of the brain circuitry that modulates negative emotional reactions to threatening environments. Collectively, our findings showed that stress exposure results in a selective elevation of p35 in BLA, and a concomitant development of an anxiogenic state. The present data support the notion that the activation of Cdk5/p35 in BLA may be involved in the cascade of molecular events ultimately leading to the emergence of excessive anxiety, among others, following experiencing an unavoidable stressful event. The elucidation of such events is highly relevant for the understanding of both the adaptive responses under conditions of stress and the maladaptive consequences that culminate in affective psychopathologies. Furthermore, an improved comprehension of the molecular processes that contribute to the manifestation of stress-induced anxiety disorders may facilitate the development of more effective treatments.

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Contributors

E. Bignante performed all the experiments and statistical analysis. V. Molina designed this study. E. Bignante, G.Paglini and V. Molina performed the analysis of the data and the final manuscript. All authors have approved the final manuscript.

Conflict of interest

The authors declare that, except for income received from our primary employer, no financial support or compensation has been received from any individual or corporate entity over the past three years for research or professional service and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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