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Involvement of septal Cdk5 in the emergence of excessive anxiety induced by stress

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Abstract

The aim of the present study was to evaluate whether the activation of Cdk5, a protein that has been suggested to participate in higher cognitive functions, is required for the onset of a sensitized anxiety-related behavior induced by stress.

The exposure to restraint enhanced both Cdk5 expression in certain subareas of the septohippocampal system, principally in the lateral septum (LS) and septal Cdk5 kinase activity in rats. Behaviorally, restrained wild type mice showed a behavior indicative of enhanced anxiety in the elevated plus maze (EPM). In contrast, unstressed mice and stressed knockout mice, which lacked the p35 protein, the natural activator of Cdk5, displayed similar anxiety-like behavior in the EPM. Finally, the intra-LS infusion of olomoucine – a Cdk5 inhibitor – blocked the enhanced anxiety in the EPM induced by prior stress in rats. All these data provide evidence that septal Cdk5 is required in the emergence of a sensitized emotional process induced by stress.

1. Introduction

The emotional response to a novel innocuous or to a mildly stressful situation is critically influenced by previous experience with threatening stimuli. For instance, animals exposed to diverse types of stressful experiences react later on with long lasting and exaggerated fear and anxiety when subsequently faced with novel situations that would normally induce minimal emotional disturbances (Martijena et al., 1997, 2002; Stam et al., 2000; Stam, 2007; Wiedenmayer, 2004; Rodriguez Manzanares et al., 2005; Adamec et al., 2005; Calfa et al., 2006). Therefore, prior stress results in excessive negative emotional reactions to novel environmental demands, even when the novel experience is not strictly related to the original situation. This process has been tentatively defined as stress or emotional sensitization

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(Rosen and Schulkin, 1998; Stam et al., 2000; Wiedenmayer, 2004; Calfa et al., 2006, 2007). There is a growing interest in animal models of stress, since they can model the impact that stress can produce on different aspects of affect and emotion in humans. In fact, sensitized fearfulness is a feature of post-traumatic stress disorders (PTSD) with the hyperreactivity to a novel stimulus after short-lasting but intense stress exposure having phenomenologic aspects that resemble PTSD in humans (Pitman, 1997; Stam, 2007).

The septohippocampal system is involved in the processing of aversive information (Gray and McNaughton, 2000). The septal region, in particular, has been inferred to participate in the modulation of processes related to higher cognitive functions, including learning and memory. Moreover, this region modulates the behavioral outcome after stressful experiences (Jakab and Leranth, 1995). The lateral septum (LS) plays an important regulatory role in the occurrence of diverse affective states (Sheehan et al., 2004), integrating cognitive information from cortical areas, and emotional information arriving via the amygdala. This information is later relayed to diencephalic and mesencephalic regions to adjust behavior to changing environmental demands. Regarding fear and anxiety, this brain structure controls fear-related behavior, principally by inhibiting behavioral reactions to stressful events (Sheehan et al., 2004). The activation of LS neurons following aversive stimulation may result in the attenuation of the psychological severity of the stress (Sheehan et al., 2004; Yadin and Thomas, 1996; Thomas and Evans, 1983). Consistent with this notion, animals subjected to electrolytic or exitotoxic lesions, or with pharmacological inactivation of this brain region (Treit and Pesold, 1990), showed a behavioral response comparable to that observed following the administration of anxiolytic compounds in experimental paradigms of anxiety (Pesold and Treit, 1992, 1994) such as the elevated plus maze (EPM). Alternatively, it was proposed that the decreased reactivity to fear and anxiety produced by septal lesion could in fact be due to a shift from active to passive coping strategies (Sheehan et al., 2004).

Recently, we suggested that the LS plays an essential role in the emergence of a sensitized emotional process induced by continuous stress. Rats previously subjected to an inescapable stressful experience exhibited excessive anxiety-like behavior when tested in the EPM (Calfa et al., 2006, 2007).

Although copious information is available regarding behavioral data, the molecular and cellular mechanisms underlying this sensitized process triggered by stress are still unclear. This is a relevant issue since the understanding of the molecular mechanism involved in the behavioral response to environmental threats could provide an insight into the mechanism involved in the onset of emotional disorders.

Cyclin-dependent kinase 5 (Cdk5), a proline-directed serine/threonine kinase, has kinase activity only in the nervous system, and requires the binding of neuronal specific activator proteins, p35 or its isoform p39. This enzyme plays an important role in the normal brain development regulating neuronal migration, axodendritic organization, laminar architecture (Dhavan and Tsai, 2001; Paglini and Cáceres, 2001) and in diverse synaptic processes (Fletcher et al., 1999; Paglini et al., 2001; Tomizawa et al., 2002).

Recent reports have suggested that Cdk5 is involved in associative learning and memory (Dhavan et al., 2002; Fischer et al., 2003, 2005; Ohshima et al., 2005, Angelo et al., 2006). For instance, increased Cdk5 activity in the LS is associated with contextual fear conditioning and stress, whereas the pharmacological blockade of Cdk5 activity in the LS inhibits fear learning (Fischer et al., 2002). Moreover, a pivotal role in synaptic plasticity linked to learning and memory was also proposed (Li et al., 2001; Wei et al., 2005; Fischer et al., 2005). In summary, the up-regulation of this protein could be involved in the process leading to the storage of environmental information, including aversive experiences.

Therefore, the main aim of this study was to evaluate whether the activation of Cdk5 in the septohippocampal system is required for the development of the sensitized anxiety-related behavior induced by prior exposure to an unavoidable stressful situation.

2. Experimental procedures

2.1. Animals

Adult male Wistar rats (270–320 g) of our breeding were housed in standard laboratory Plexiglas cages (three to four per cage, dimension of the cage: 30×45×18 cm) with food and water *ad libitum*. Rats were habituated to manipulation during the week before the experiments.

For some experiments, we used previously characterized transgenic mice (Chae et al., 1997). Specifically, we used adult (8–12 week old) male mutant $p35^{-/-}$ and wild type $p35^{+/+}$ mice (40–50 g) maintained in a 129/SvJ×C57BL/6J background via brother–sister matings, and housed in standard laboratory Plexiglas cages (six to eight per cage, dimension of the cage: 30×45×18 cm) with food and water *ad libitum*.

All animals were maintained throughout the experiments in a 12 h light/dark cycle (lights on at 7:00 a.m.) with a constant room temperature of 21±2 °C; behavioral testing was performed during the light cycle between 10:00 a.m. and 2:00 p.m. Procedures were conducted in accordance with the National Institute of 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 approved 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. Stress

Animals were transferred to the experimental room and placed inside a plastic restrainer fitted closely to body size which did not allow the animal to move. The restrainer had numerous holes to allow accessibility of fresh air, with only the tail and the tip of the nose free (Cancela et al., 1988). At the end of the stress session, animals were returned to the colony room (Group RES). Control animals were transferred in their own home cages to the experimental room, handled for 2 min and then returned to the colony room (Group CON).

2.3. Immunohistochemical analysis

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 then sectioned in a cryostat into 30 μm thick coronal slices. Sections were immersed in 0.1 M PBS and later incubated for an hour in a solution of 10% methanol, 3% hydrogen peroxide in PBS to eliminate the endogenous peroxidase activity. Later, sections were

incubated for 1 h in a blocking solution (5% bovine-serum albumin (BSA) and 0.3% Tritón X-100 in 0.1 M PBS) and then incubated for 48 h at 4 °C with polyclonal Cdk5 antibody (C-8) or p35 antibody (C-19) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 1:400 in 0.1 M PBS containing 1% BSA and 0.1% Tritón X-100. Subsequently, the sections were washed and incubated at room temperature with biotinylated 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): sections were incubated for 5 min with a solution containing 0.05% of DAB and 0.0006% of hydrogen peroxide. Brain sections were mounted onto slice glass, dehydrated and coverslipped prior to viewing with a light microscopy.

2.4. Quantification

Positive Cdk5 or p35 cells were identified using light field microscopy (Zeiss Axioplan) with Metamorph computer software at a magnification of 200× and then counted with computational software (a SCION program from the NIH). The quantification was carried out by a person not involved in the experiment and performed using an identical size area (0.16 mm^2) of the same shape. The anteroposterior (AP) coordinates from Bregma (Paxinos and Watson, 1997) of the lateral septum included for detailed analysis were AP: 1.20 to 0.20.

2.5. Assays for Cdk5 activity

Rats were killed by decapitation and brains were removed and chilled in ice-cold saline. The septum was dissected and homogenized in a lysis buffer RIPA (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 100 μg/ml PMSF, 1 μg/ml pepstatin, and 0.2 mM sodium orthovanadate). After centrifugation, 250 μg of total cellular proteins from supernatants were immunoprecipitated with an anti-Cdk5 antibody conjugate to agarose beads (C-8, Santa Cruz Biotechnology, Santa Cruz, CA, USA), as described previously (Pigino et al., 1997) and 30 μg of the same total cellular proteins from supernatant were used to perform western blots in order to confirm the presence of Cdk5 protein in the sample (routine control). Cdk5 in vitro kinase assays were carried out as described in several works of our laboratory (Pigino et al., 1997; Paglini et al., 1998; Paglini et al., 2001). Briefly, immunoprecipitates were washed three times with RIPA and once with kinase buffer (30 mM MOPS, pH 7.2, 5 mM MgCl2, and 0.1 mM cold ATP). The washed beads were then incubated with kinase buffer containing 1.5 μg of histone H1, as a Cdk5 substrate, and 5 μCi of [γ-32P] ATP in a final volume of 30 μl. After 30 min of incubation at 30 °C, 30 μl of 2× Laemmli sample buffer (Laemmli, 1970) was added to each sample to stop the reaction, and they were then analyzed by SDS-PAGE using 12% acrylamide gels. Once gels were dried, radiographic bands were visualized in a phosphoimage instrument (Storm 840, Molecular Dynamics) and quantified with the Image Quant software (Molecular Dynamics).

2.6. Elevated plus maze

The elevated plus maze (EPM) consisted of a plus-shaped apparatus of black Pexiglas with two opposite open arms (50×10 cm) and two opposite closed arms (50×10×40 cm). The arms were attached to a central square (10 cm^2) and the whole apparatus was elevated 50 cm above the floor. The testing room was quiet and dimly lit. Each rat was tested for 5 min and the maze was carefully cleaned after each rat with a 20% ethanol solution. The scores evaluated were number of entries into the open and closed arms and the time spent in each arm. An entry was defined as when all four paws of the rat were inside an arm. As usual, the time spent in open arms relative to the total time spent in both open and closed arms was used as an index of anxiety (Cruz et al., 1994; Martijena et al., 2002; Calfa et al., 2006). All rats were tested in the EPM 24 h after the end of the restraint experience since a number of reports, including those from this laboratory, have shown that changes in anxiety-like behavior induced by stress are still present one day after the aversive experience (Grahn et al., 1995; Calvo et al., 1998; Korte and De Boer, 2003).

For mice, the EPM was made in gray wood with two opposite open arms (30×5 cm) and two opposite closed arms (30×5×16 cm). The maze was placed at a height of 40 cm above the floor. The behavioral procedures used were similar to those described for rats, but mice were transported to the testing room under red light, habituated for 1 h and then tested 30 min after the aversive stimulus since alterations in the behavior displayed in the EPM by stressed mice are frequently observed within 30–60 min after the end of the stressful experience (Schaefer et al., 2000; Hata et al., 2001; Hsu et al., 2007).

2.7. Implantation of guide cannulae for intracerebral infusions

Rats were anesthetized with an intraperitoneal injection of ketamine (55 mg/kg)–xilazine (11 mg/kg) and placed on a stereotaxic instrument (Stoelting Inc., Wood Dale, IL) with the incisor bar set at −3.3 mm. The scalp was incised and retracted and small burr holes were drilled into the skull using a dental drill. Bilateral cannulae guides (22-gauge stainless steel tubing) were bilaterally implanted into the LS (using the following coordinates: AP: +0.2 mm, L: ±0.7 mm, and DV: −3.2 mm from the skull according to the atlas of Paxinos and Watson, 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. Animals received an

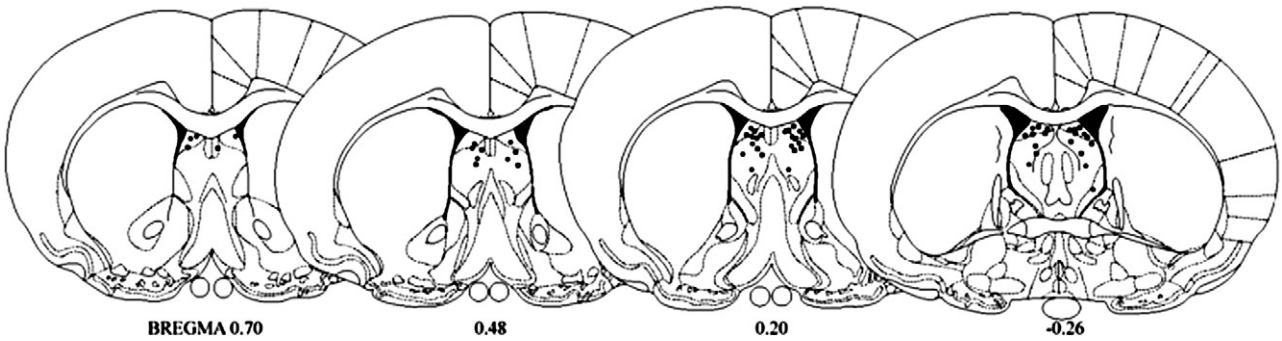

Figure 1 Placement of infusion cannulas. Schematic drawings of coronal sections of the rat brain showing the approximate location of the site of infusion of olomoucine (■) and isolomoucine (●). These drawings were adapted from Paxinos and Watson (1997).

antibiotic the first 3 days after surgery to prevent infection. After surgery, animals were gently handled every day, replacing missing dummy when necessary. The rats were allowed at least a week for post-surgical recovery and familiarized with the intracerebral infusion procedure and handled for 3–5 min in order to minimize non-specific stress responses during the experiment.

2.8. Drugs and drug administration

A solution of olomoucine or isolomoucine (Calbiochem, San Diego, CA) dissolved in DMSO was diluted with PBS to a final concentration of 20 ng/0.5 μl. 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).

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

2.9. Histology

Cannulated rats were killed by an overdose of choral hydrate, and their brains were removed and fixed in a 4% formalin solution. Coronal sections were cut in a cryostat (Leica, Nussloch, Germany) for the evaluation of the injection sites and the extent of tissue damage under a light microscope. Only those animals with the injection site in the LS were considered for statistical analysis (Fig. 1).

2.10. Statistical analysis

Results were expressed as mean ± SEM. Significant ANOVAs were followed by post hoc Newman–Keuls analysis to enable specific group comparison ($p<0.05$ was regarded as significant).

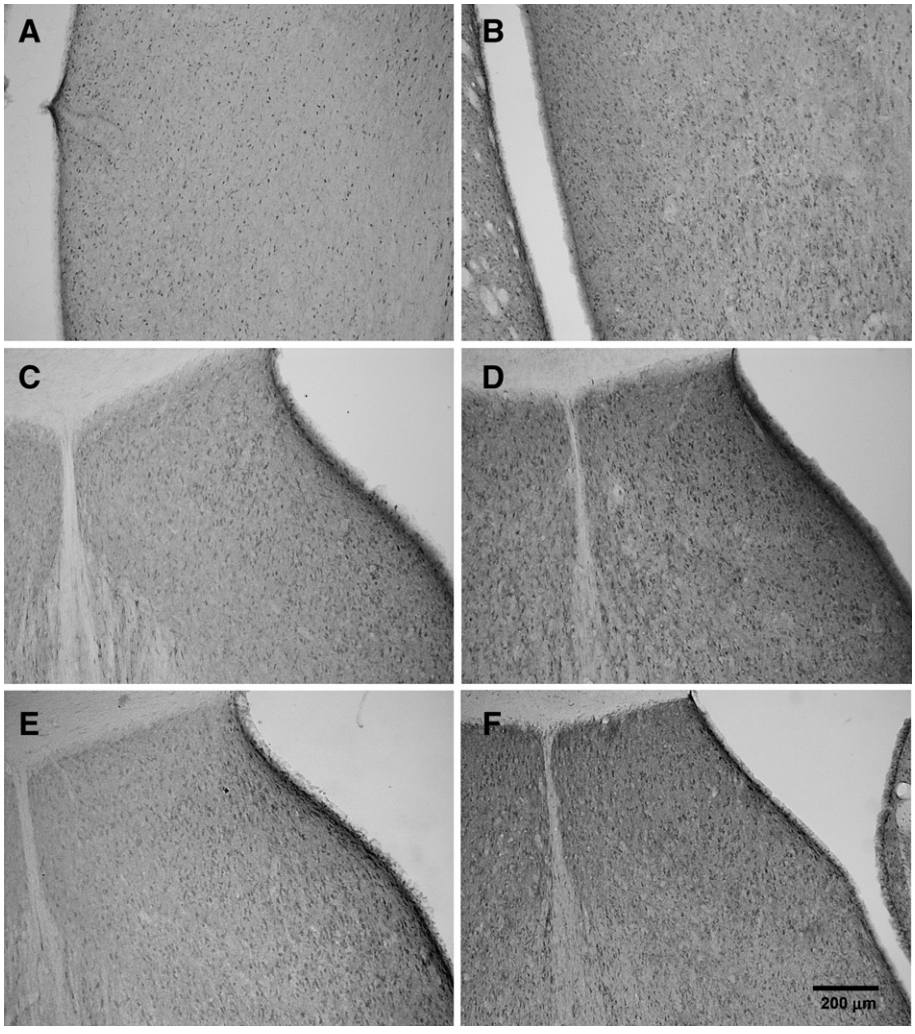

Figure 2 Photomicrographs of Cdk5 and p35 immunoreactivity (100×). Cdk5 immunoreactivity in A lateral septum ventral (LSV) from control rats and, B lateral septum ventral (LSV) from restrained rats, C lateral septum dorsal (LSD) from control rats and, D lateral septum dorsal (LSD) from restrained rats. p35 immunoreactivity in E lateral septum dorsal (LSD) from control rats and, F lateral septum dorsal (LSD) from restrained rats (DAB stain).

2.11. Experimental design

2.11.1. Experiment 1. Effect of restraint on the Cdk5 expression in the septohippocampal system

This experiment was designed to examine whether a single restraint session modified the expression of Cdk5 in different subareas of the septohippocampal brain system. Rats were subjected to a 1 hour restraint session (group RES) and sacrificed immediately. Another group of rats remained in the colony room without any experimental manipulation (group CON). Brains were removed, sectioned and submitted to the immunohistochemical procedure with anti-Cdk5 or anti-p35 antibodies. The sacrifice was carried out immediately after the immobilization based on the temporal curve of expression of Cdk5 previously reported (Fischer et al., 2002).

2.11.2. Experiment 2. Effect of restraint on Cdk5 activity in the septum

The aim of this experiment was to explore whether a change in the expression of the Cdk5 protein induced by stress, is concomitant with changes in its kinase activity. A group of rats was restrained (group RES) and immediately sacrificed. Control rats (group CON) were sacrificed without aversive stimulation. The brains from all rats were removed, then the septum was dissected for immunoprecipitation and kinase assays performed as described previously.

2.11.3. Experiment 3. Effect of restraint on the behavior in the EPM of KO and WT mice

In the current experiment, we assessed the role of an active form of Cdk5 in the generation of the emotional sensitized response, using knockout (KO) mice which lacked the p35 protein, the natural activator of Cdk5

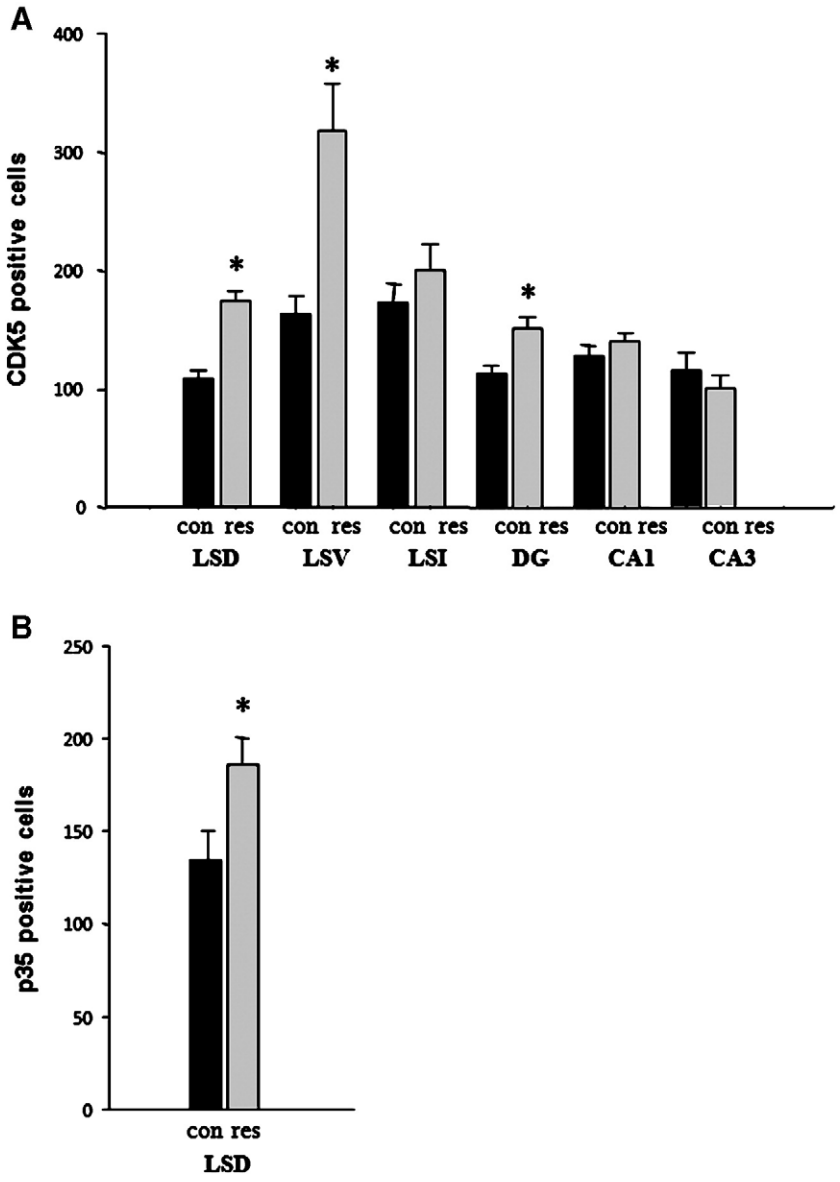

Figure 3 Cdk5 and p35 positive cells in different subareas of the septum and the hippocampus followings stress exposure. A. Quantification of Cdk5 positive cells in dorsal, ventral and intermediate portions of lateral septum (LSD, LSV, LSI; respectively), medial septum (MS) and dentate gyrus (DG), CA1 and CA3 portions of hippocampus. B. Quantification of p35 positive cells in dorsal portion of lateral septum (LSD). The values represent number of positive cells stained with DAB in an area of 0.16 mm^2 (mean ± SEM) ($n = 4$).

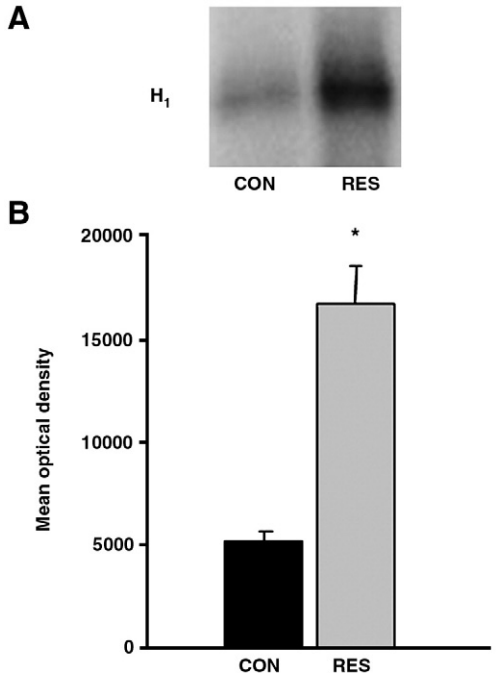

Figure 4 Effect of restraint on Cdk5 activity of septum lysates. A. Representative autoradiogram indicating a higher phosphorylation of H1 by Cdk5 immunoprecipitated from the septum of stressed (RES) as compared to control (CON) rats. B. Mean optical density corresponding to autoradiogram shown in A. Data represent the mean ± SEM of the optical density. *$p<0.001$ vs. control group ($n=4$).

(Lew et al., 1994; Tsai et al., 1994). We investigated the anxiety-like response in a group of mutant $p35^{-/-}$ which presented a deficiency in the kinase activity of Cdk5 (Group KO-RES) and also a group of wild type ($p35^{+/+}$) mice (Group WT-RES), which were restrained for 1 h and submitted to the EPM half hour later. Additionally, a group WT and a group KO mice without stress were maintained in their home cages and later tested in the EPM (Group WT and KO-CON).

2.11.4. Experiment 4. Effect of local infusion with olomoucine into the LS on the behavior displayed in the EPM by restrained rats
To verify the functional role of Cdk5 on the LS in the emotional sensitization process, we performed blocking experiments with local infusion of olomoucine (20 ng/0.5 μl), a Cdk5 inhibitor, (Veselý et al., 1994; Meijer, 1995; Bibb et al., 2001) or with isolomoucine, an analogue that lacks blocking properties on Cdk5 activity. Rats were cannulated in the LS and distributed randomly into four groups: OLO-CON (animals infused with olomoucine without restraint); OLO-RES, (rats infused with olomoucine and restrained for 1 h); ISO-CON, (rats infused with isolomoucine without restraint) and ISO-RES, (rats infused with isolomoucine and restrained for 1 h). Stressed rats were infused with either olomoucine or isolomoucine 15 min before stress exposure. All subjects were tested in the EPM 24 h after the end of the restraint experience.

3. Results

3.1. Experiment 1. The exposure to restraint increased the expression of Cdk5 in the lateral septum and the dentate gyrus

Immunohistochemical findings showed a significant increase in Cdk5 protein levels, restricted to the dorsal and ventral sections of the lateral septum (LSD and LSV respectively) and also in the gyrus dentate (DG) of the hippocampus as a consequence of the stressful event (Figs. 2 and 3). ANOVA revealed a significant effect of stress on the number of Cdk5 positive cells in LSD ($F_{(1,19)}=32.1686$ $p<0.0001$), LSV ($F_{(1,21)}=14.6355$ $p<0.001$) and DG ($F_{(1,19)}=11.2920$ $p<0.01$) but not in the lateral septum intermediate portion (LSI), neither in the medial septum (MS) nor in the CA1 and CA3 portions of hippocampus.

Moreover, in the same groups of rats, we observed a significant increase in the levels of p35 in the LSD as a consequence of the restraint exposure (Figs. 2 and 3). ANOVA revealed a significant effect of stress on the number of p35 positive cells in LSD ($F_{(1,21)}=6{,}1469$ $p<0.05$).

3.2. Experiment 2. A session of restraint increased Cdk5 kinase activity in the septum

The increase in the expression of Cdk5 in slices of LS was correlated with a two-fold increase in its kinase activity in the septum. In fact, Cdk5 immunoprecipitated from septum lysates of stressed animals showed a significantly higher H1 histone phosphorylation as compared to septum lysates from unstressed rats (Fig. 4). ANOVA showed a significant effect of restraint on the optical density (OD) of the radiographic bands ($F_{(1,6)}=47.6554$ $p<0.0005$) (Fig. 4). In order to confirm that the H1 phosphorylation is associated with Cdk5 kinase activity, parallel Cdk5 immunoprecipitated samples were incubated with different doses of the Cdk5 inhibitor olomoucine during the assay. This activity was inhibited in a dose-dependent fashion by olomoucine (data not shown).

3.3. Experiment 3. The lack of the p35 protein prevents the emotional sensitization process induced by stress

As expected, wild type ($p35^{+/+}$) mice previously exposed to a single restraint session showed a reduction in the time spent on open arms

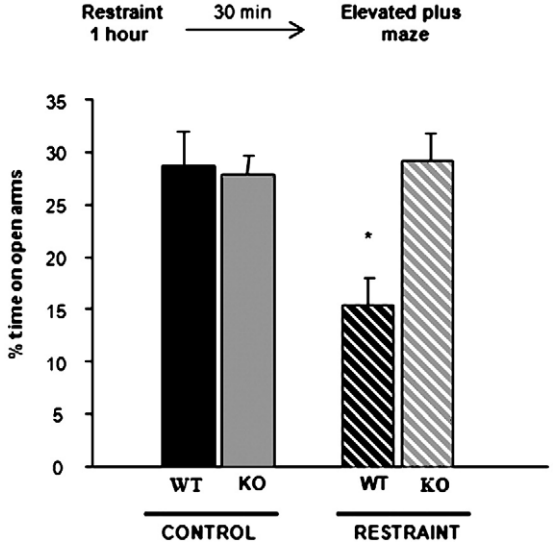

Figure 5 Effect of restraint on the percentage of time spent on open arms in the EPM in WT and KO mice. Restraint induced a reduction in the time spent on open arms in WT mice but not in KO mice. Data represent the mean ± SEM of the percentage of time spent on open arms relative to total time spent on all four arms. *$p<0.01$ vs. all other groups ($n=11-13$).

in the EPM, indicative of enhanced anxiety, (Group WT-RES). In contrast, p35$^{-/-}$ stressed mice (Group KO-RES) displayed a similar percentage of permanency in the open arms to that performed by animals without stress (Group WT and Group KO-CON) (Fig. 5).

Two-way ANOVA revealed a significant effect of condition (WT or KO) ($F_{(1,46)}=6.4554$ $p<0.05$), treatment (CON or RES) ($F_{(1,46)}=5.3819$ $p<0.05$) and the interaction of condition and treatment ($F_{(1,46)}=8.2559$ $p<0.01$) on the percentage of time spent on open arms. A post hoc Newman–Keuls analysis indicated that restraint reduced the time spent on open arms in the EPM ($p<0.01$) and this reduction was absent for the KO condition. In other words, stress did not affect the behavior performed in the EPM by p35$^{-/-}$ mice. This finding suggests that the presence of an active Cdk5 protein is required for the induction of enhanced anxiety-like behavior following stress exposure.

3.4. Experiment 4. The local infusion of olomoucine into the LS prevents the excessive anxiety induced by prior stress

Animals that experienced the stressful stimulus and were locally infused into the LS with isolomoucine (Group ISO-RES) performed later on an exaggerated anxiety-like behavior in the EPM, since they showed a clear reduction in the time spent in open arms. In contrast, OLO-infused rats submitted to stress exhibited an activity in open arms similar to that shown by non-stressed animals (Fig. 6). Two-way ANOVA showed a significant effect of treatment (CON or RES) ($F_{(1,30)}=9.6394$ $p<0.01$) and the interaction of pretreatment and treatment (ISO or OLO) (CON or RES) ($F_{(1,30)}=5.2469$ $p<0.05$) on the percentage of time spent on open arms. Furthermore, the Newman–Keuls post hoc test revealed a reduction in the percentage of time spent on open arms produced by restraint with this effect being fully prevented by the previous administration of olomoucine intra-LS ($p<0.05$).

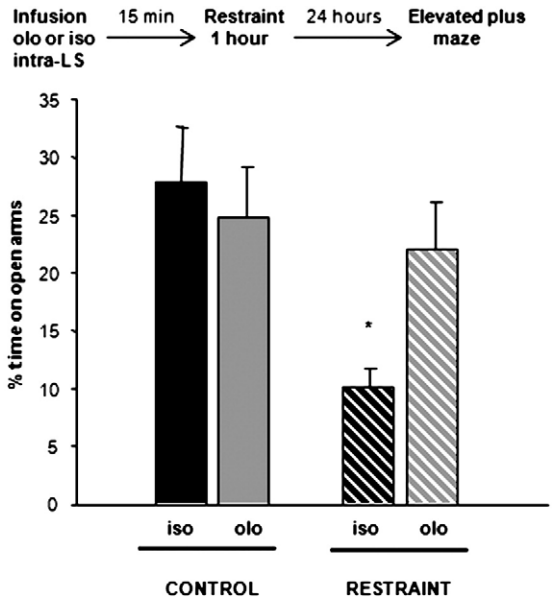

Figure 6 Effect of restraint on the percentage of time spent on open arms in the EPM: influence of previous intra-LS injection of OLO or ISO. Restrained rats exhibited a reduction in the time spent on open arms, and this effect was prevented by the local infusion of olomoucine but not of isolomoucine into the LS previous to stress. Data represent the mean ± SEM of the percentage of time spent on open arms relative to total time spent on all four arms. *$p<0.01$ vs. all other groups ($n=7–9$).

No significant effect on the number of closed arms entries was detected (ISO-CON = 9.625 ± 1.25; ISO-RES = 7.6 ± 065; OLO-CON = 6.88 ± 1.08; OLO-RES = 6.67 ± 0.66) suggesting that the excessive anxiety induced by restraint is not due to a reduction in exploratory activity (Cruz et al., 1994) (data not shown).

Additionally, in a separate experiment, we verified that rats infused with ISO performed a similar behavior in the EPM to that displayed by animals infused with vehicle (VEH). Two-way ANOVA performed on the percentage of time spent on open arms indicated a significant effect of treatment (CON or RES) ($F_{(1,32)}=38.3658$ $p<0.000001$) but no significant effect of pretreatment (VEH-ISO), or the interaction pretreatment and treatment. The Newman–Keuls post hoc analysis revealed a reduction on percentage of time spent on open arms provoked by immobilization. ($p<0.001$) (data not shown).

4. Discussion

The present results showed a selective increase in the level of the Cdk5 protein in certain subareas of the septohippocampal system, shortly after stress exposure. Moreover, the dorsal and the ventral portions of the LS were among the subareas having the strongest expression of Cdk5 in response to stress. The fact that such an enhancement was only observed in selected subareas rules out a possible unspecific general increase of this protein in the septohippocampal system associated with an increased arousal due to the aversive stimulation. Concomitant with the increased Cdk5 content in the LS, a robust enhancement in the kinase activity of this enzyme was observed in the septum of stressed rats, compared to that exhibited by unstressed animals.

Furthermore, an increase in the expression of the activator of Cdk5, p35, in the LSD was detected which correlates with the enhanced kinase activity induced by the stress experience. Collectively, these findings suggest a potential role of Cdk5 in the emergence of the emotional sequelae resulting from aversive experiences, since the LS is a primary brain region involved in the induction of a sensitized emotional process (Calfa et al., 2006, 2007) and in the modulation of the behavioral flexibility required after environmental pressures (Mongeau et al., 2003). This prediction was confirmed by our behavioral experiments. In fact, unlike the enhanced anxiety shown by stressed wild type mice, animals lacking the Cdk5 activator subjected to restraint were behaviorally similar in the EPM to both the unstressed wild type and p35 KO mice, indicating that the absence of Cdk5 activity attenuated the usual increase in the anxiety-like behavior produced by the inescapable stressful stimulus. However, since p35 KO animals display neuronal migration deficits and aberrant circuits we cannot rule out that these abnormalities contribute to the behavioral phenotype (Chae et al., 1997; Patel et al., 2004; Ohshima et al., 2005). Consistent with the notion that Cdk5 activation participates in the emotional sequelae induced by stress, the local infusion of a Cdk5 inhibitor in the LS reduced the decrease in the time spent in open arms induced by stress in rats, a behavioral pattern thought to reflect enhanced anxiety. Moreover, this behavioral profile shows that restraint resulted in a clear decrease of the conflict approach/avoidance behavior

displayed during the EPM. Interestingly, this type of behavioral response is anxiolytic sensitive (Albrechet-Souza et al., 2007) and has been the principal basis for the pharmacological validation of the EPM as an animal model of anxiety. Other behaviors displayed in the EPM such those interpreted as risk assessment were not evaluated in the current work since they are indicative of other functional significance than the classic conflict of approach/avoidance behavior (Anseloni and Brandão, 1997; Albrechet-Souza et al., 2007). Furthermore, it was previously shown that stress exposure does not modify risk assessment postures when the EPM test was carried out one day later (Calfa et al., 2007).

A vast amount of experimental evidence has been published in support of an excessive emotional reaction to mild or innocuous stimuli in rodents that previously experienced different types of aversive situations. In fact, a single exposure to a variety of stressors subsequently resulted in excessive anxiety-like behavior in the EPM both in mice and rats (Martijena et al., 1997, 2002; Hata et al., 2001; Korte and De Boer, 2003; Calfa et al., 2006). Such a process has been tentatively defined as stress sensitization or emotional sensitization (Rosen and Schulkin, 1998; Stam et al., 2000; Stam, 2007; Wiedenmayer, 2004; Calfa et al., 2006).

Collectively, our results, using different experimental approaches, provide compelling evidence that the activation of Cdk5, preferentially in the LS, is an important mechanism required for the induction of a sensitized emotional process triggered by stress.

The neurons of the LS are predominantly GABAergic projection neurons, which are highly and reciprocally interconnected with diverse brain areas involved in the modulation of fear and anxiety. In fact, inputs from different sources arrive to different segments of these spiny GABAergic neurons of the LS, and in turn these neurons project to brain areas outside the septum (Jakab and Leranth, 1995) which are implicated in diverse aspects of the complex response to stress (Desmedt et al., 1998; Thomas and Sancar, 2001). It is important to indicate that LS interacts with brain areas directly involved in fear regulation and changes in the LS were suggested to be a down-stream mechanism by which these areas control fear (Sheehan et al., 2004). In line with this view, it is tempting to speculate that the up-regulation of Cdk5 in the LS induced by stress could be part of this mechanism.

The facilitating influence of prior stress on the activation of Cdk5 in the LS could have a major adaptive role, improving survival capability in the face of future threats. Consonant with this reasoning, the enhanced activation of this enzyme induced by a previous restraint experience could be important in the cascade of events that leads to a permissive role for the emergence of increased anxiety-like behavior in response to subsequent environmental demands. In fact, it is widely known that increased neuronal activity within the LS after different stressful stimuli facilitates the occurrence of fearful behaviors in response to such stimuli (see Sheehan et al., 2004).

Coincidentally with the current results, previous reports have demonstrated a robust activation of septal Cdk5 in mice in response to a stressor similar to that used in this study (Fischer et al., 2002). Moreover, these authors showed that the local infusion of butyrolactone I, another Cdk5 inhibitor, in the LS fully prevented the facilitation of restraint on contextual fear conditioning (Fischer et al., 2002). Therefore, prior findings, as well as those reported in this study, suggest that the emergence of stress-induced negative emotional reactions (increased fear and excessive anxiety) requires the up-regulation of Cdk5 in LS.

According to previous research, a role of Cdk5 in higher cognitive functions specifically related to contextual associative and extinction learning as well as in spatial memory has been proposed (Fischer et al., 2002, 2005; Sananbenesi et al., 2007). In accordance with this proposal, a functional role of this protein in synaptic plasticity related to learning was also described (Fischer et al., 2005; Angelo et al., 2006). In our experimental paradigm, the activation of septal Cdk5 is required for the induction of a stress-induced sensitized emotional process which is non-associative learning, since the behavioral effect observed in the current report is due to the sensitizing action of the aversive stimulus (restraint) with there being no relation between the aversive experience and the subsequent situation (EPM). Hence, in addition to its role in associative learning as previously suggested, Cdk5 could also play a role in non-associative learning. The participation of a sensitized non-associative component in classic Pavlovian fear learning using contextual cues has been previously proposed (Kamprath and Wotjak, 2004). Therefore, the up-regulation of septal Cdk5 described as a consequence of contextual fear learning might be due, in part, to a non-associative component. Further experiments are necessary in order to analyze this possibility.

Alternatively, the activation of Cdk5 in LS might represent a mechanism independently implicated in both associative and non-associative learning processes. Indeed, the acquisition and the storage of emotionally relevant information promote a cascade of events that has a decisive influence on the behavioral outcome exhibited in the face of future environmental demands. With this line of reasoning, the up-regulation of this protein in the septal area could be an important component in this cascade of events that ultimately leads to the onset of such a process.

To date, the neural mechanism involved in the emergence and persistence of exaggerated emotional reactions following stress, and how these reactions are mediated at the neurotransmitter level, are still poorly understood. Although changes at multiple neurotransmitter systems are known to participate in the complex set of behavioral reactions following stress, the possible selective involvement of a particular neurotransmitter or neurotransmitters in the induction, further development or expression of this sensitized process remains to be fully established. Adamec et al. (1999) using a predator paradigm to induce behavioral sensitization, demonstrated that blockade of NMDA sites prevented the induction of such sensitized process, but not the consolidation of the aversive experience, suggesting that a NMDA-mediated mechanism is necessary for the acquisition of such experience. Moreover, it is widely accepted that activation of NMDA sites are crucially involved in the acquisition of fear conditioning, either using contextual or discrete cues (Fanselow and Kim, 1994; Lee and Kim, 1998; Castellano et al., 2001; Walker and Davis, 2002). Finally, NMDA receptors have been strongly implicated in mechanisms underlying learning and memory (Riedel et al., 2003). In conclusion, all this evidence supports the view that a NMDA-mediated mechanism in selected brain areas is an essential requirement for the acquisition of new and relevant environmental information either through associative or non-associative processes or by both.

Interestingly, Cdk5 phosphorylates the NR2A subunit of the NMDA receptor affecting NMDA-regulating synaptic transmission.

Moreover, the inhibition of this enzyme resulted in the prevention of LTP induction and NMDA-evoked currents (Li et al., 2001), indicating that activation of Cdk5 is associated with a facilitated glutamatergic neurotransmission acting on NMDA receptors. Therefore, if NMDA is involved in the emergence of an emotional sensitized process induced by stress, as proposed by Adamec et al. (1999), and Cdk5 also facilitates NMDA-mediated effects, we can tentatively suggest that the involvement of this protein in the promotion of emotional sensitization triggered by stress might be mediated by NMDA sites. Obviously, since Cdk5 exerts a critical role in a number of synaptic functions, this protein could be also participating in the wide number of changes at diverse neurotransmitters reported following stress.

It is known that stress-related hormones such as glucocorticoids participate in the emotional consequences provoked by aversive stimulation (De Kloet et al., 1999; Korte, 2001). In fact, the activity of septal glucocorticoid receptors is functionally linked with the onset of a sensitized emotional reaction to stress (Calfa et al., 2006). Moreover, a recent report proposed that Cdk5 acts by modulating glucocorticoid receptor transcriptional activity (Kino et al., 2007). Therefore, an alternative explanation for the participation of Cdk5 in the emotional sequelae after stress could involve, in part, the effect of this protein on these receptors.

All together, these data support the view that the activation of septal Cdk5 is required for the onset of a sensitized emotional process after stress. Specifically, the present research described the effect of a prior restraint experience on Cdk5 expression and activity in the LS and on the behavior performed in an animal model of anxiety. Moreover, depending on the emotional past experiences of an organism, the promotion of behavioral flexibility with changing environmental conditions could have a major adaptive role in face of new threats. Consistent with this notion, the failure to regulate Cdk5 activity or the hyperactivity of Cdk5 in response to irrelevant stimulus, may contribute to the emergence of an excessive negative emotional state related to the experience of intense stressful life events. Furthermore, the comprehension of the molecular mechanisms involved in the behavioral response to threatening experiences could give insight into the mechanisms involved in the onset of anxiety-related disorders such as PTSD and could indicate potential targets for psychotherapeutic drugs development.

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Contributors

E. Bignante performed all the experiments and statistical analysis. P. Rodriguez, M. Bertotto, E. Mlewski and D. Bussolino collaborated with the experiments. 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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