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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.


## 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