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Research report

Increased nitric oxide-mediated neurotransmission in the medial prefrontal cortex is associated with the long lasting anxiogenic-like effect of predator exposure

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HIGHLIGHTS

- Predator threat stress induces long-lasting activation of nitrergic system.
- Predator exposure produces a long lasting increase in neuronial isoform of nitric oxide synthase in prefrontal cortex.
- Predator exposure produces an increase in NO production in prefrontal cortex.

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ABSTRACT

Posttraumatic stress disorder (PTSD) is an anxiety disorder caused by the experience of a severe traumatic event. In rats this disorder has been modeled by exposure to a predator threat. PTSD has been associated to structural and functional changes in the medial prefrontal cortex (mPFC). Direct injections into this brain region of glutamate antagonists or inhibitors of the nitric oxide synthase (NOS) enzyme cause anxiolytic-like effects in rodents. In the present work we investigated if the behavioral changes induced by predator exposure are associated with changes in the mPFC nitrergic system. Since the hippocampus, amygdala and dorsal periaqueductal grey have also been associated to anxiety disorders, including PTSD, we also verified if this procedure would modify the nitrergic system in these regions. Male Wistar rats were exposed to a dummy or live cat for ten minutes and tested in the elevated plus maze test (EPM) seven days later. Immediately after the test their brains were removed for neuronal NOS (nNOS) immunohistochemistry detection and measurements of nitrite/nitrate (NO_x) levels. Exposure to the live cat increased freezing responses. One week later the animals that froze when confronted with the cat presented a decreased percentage of entries in the open arms of the EPM and an increased number of nNOS positive neurons in the mPFC and basolateral nucleus of amygdala, but not in the hippocampus, central and medial nuclei of amygdaloid complex or dorsal–lateral periaqueductal grey. Moreover, cat exposed animals showed increased NO_x levels in the mPFC but not in the hippocampus one week later. The number of nNOS neurons and NO_x levels in the mPFC showed a significant correlation with freezing time during cat exposure. Our results suggest that plastic modifications of the nitrergic system in the mPFC could be related to long lasting behavioral changes induced by severe traumatic events such as predator exposure.

1. Introduction

Posttraumatic stress disorder (PTSD) is a debilitating mental illness characterized by recurrent distressing memories of an initial traumatic event, emotional numbing and hyperarousal [1]. In humans, typical symptoms of PTSD include intrusive memories, dreams, images and thoughts related to an initial traumatic experience [1]. Several limbic areas are proposed to mediate these behavioral changes. Although the precise biological mechanisms

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involved remain unclear, recent studies suggest that an interaction between the ventromedial prefrontal cortex (vmPFC), hippocampus and amygdala is critically involved in the pathogenesis of PTSD [2,3].

Human imaging studies have shown that PTSD patients usually present hypoactivity of the vmPFC, reduced hippocampal volume and amygdala hyperactivity [4,5]. The role of the hippocampus in episodic memory [6] and of the amygdala in fear and anxiety has been previously established [7]. The vmPFC is thought to regulate emotional responses by directly inhibiting amygdala activity [8,9]. PTSD, therefore, could result from a lack of vmPFC inhibitory control and deficient hippocampal function resulting in increased amygdala output.

Selective serotonin reuptake inhibitors (SSRI), first-line drugs for the treatment of PTSD, prevent the long lasting anxiogenic effects induced by traumatic events such as predator exposure [10,11]. SSRIs, however, are not always effective in PTSD and the clinical response depend on repeated treatment for several weeks [12], indicating that new therapeutic approaches are needed. Nitric oxide (NO) is an atypical neurotransmitter produced on demand from the amino acid L-arginine by a family of enzymes called NO synthases (NOS). It is a short-lived gas, highly liposoluble, released from postsynaptic neuronal membranes [13]. The neuronal NOS (nNOS) isoform is constitutive in the central nervous system (CNS) and located in specific brain regions [13], where it is intimately associated with glutamate NMDA receptors by the PSD95 protein [14,15]. NO can diffuse over a significant distance and influences target cells far away from its neuronal source [16]. It acts primarily as an intercellular messenger by activating soluble guanylate cyclase (sGC) [17] and modulating the release and effects of glutamate and other neurotransmitters such as serotonin and GABA [14]. NO is proposed to play a role in several brain functions and dysfunctions such as neuronal excitability, synaptic plasticity, neurotoxicity and neuroprotection [18]. It can also modulate anxiety-like behavior. The nNOS enzyme is located in key brain regions associated with defensive responses, including the amygdala, hippocampus, vmPFC and dorsolateral periaqueductal grey (dlPAG) [13]. The number of nNOS expressing neurons increases in anxiety-related brain areas after restraint stress [19], an anxiogenic stimulus [20]. Systemic administration of drugs that act on NO mediated neurotransmission modifies anxiety- and depressive-like behaviors [21,22].

More recently, an association was found between a genetic variation of NOS1AP gene and the risk for PTSD development [23]. Thus, the present work investigated the hypothesis that the long lasting anxiogenic alterations induced by predator exposure are associated [24] with activation of nitrergic mediated neurotransmission within brain areas related to PTSD (the vmPFC, amygdala, hippocampal formation and dlPAG, a key structure in defensive responses) [25].

2. Materials and methods

2.1. Animals

Male Wistar rats ($n = 22$) weighing 210–230 g were used in the present study. Animals were obtained from the colony of Animal Care Unit of the Pharmaceutical Science School/University of Sao Paulo. Rats were housed in groups in plastic cages until the beginning of the experiments. Then, they were house individually until the end of the behavioral tests. All animals were maintained in a temperature controlled room (24 ± 2 °C) with free access to food and water and under a 12 h light/dark cycle (lights on at 7 a.m.). All behavioral analyses were performed during the light part of the cycle. A male cat (weight 3 kg), originated from the Animal Care Unit of the University of São Paulo, was used in the present work. A toy cat with similar characteristic of the live animal was used as the control group (dummy). The Institution's Animal Ethics Committee approved the housing conditions and all experimental procedures (protocol number 137/2007).

2.2. Apparatus

2.2.1. Predator exposure box

The predator exposure box consisted of a rectangular arena (80 cm × 22 cm × 50 cm) with Plexiglas walls and a metal grid floor as previously describe [26]. The box was designed to comfortably contain the cat and to provide enough space for measuring the rat location (proximal or distal) to the cat compartment. The apparatus was located in a sound-attenuated 40 W illuminated room. It was covered by a Plexiglas transparent roof and divided into two opposed compartments of the same size (40 cm × 22 cm × 50 cm) by a metal grid wall that allowed rats to see and smell the cat. In the experimental session each rat was placed in the middle of the rat compartment always facing the compartment that contained the live or dummy cat.

2.2.2. Elevated plus maze test (EPM)

The wooden apparatus had two opposite open arms (50 cm × 10 cm) crossed at a right angle by two arms of the same dimensions enclosed by 40-cm-high walls with no roof. The maze was located 50 cm above the floor, and a 1-cm high Plexiglas edge surrounded the open arms to prevent falls. The experiment took place in a sound attenuated temperature-controlled (24 ± 1 °C) room, illuminated by three 40-W fluorescent bulbs placed 4 m above the apparatus. Rodents naturally avoid the open arms of the EPM, and anxiolytic compounds typically increase the exploration of these arms without changing the number of enclosed arm entries [27,28]. The Anymaze software (Stoelting Co., Wood Dale, USA) was employed for behavioral analysis. It detects the position of the animal in the maze and calculates the number of entries and time spent in open and enclosed arms.

2.3. Experimental design

2.3.1. Experiment 1

All behavioral procedures were carried out during the light cycle (8 a.m.–12 p.m.). Rats were handled during 5 min by the experimenter and habituated to the predator exposure box for 10 min on days one and two. Handling involved picking up the rat with a gloved hand under the forepaws. The rat was held and supported on the forearm of the handler. The grip was loose unless the rat tried to escape, when it was tightened until the rat became quiet. On day three the animals were placed into the predator exposure box and exposed to the dummy or live cat for 10 min. During this period the session was videotaped for posterior analysis of total time spent in freezing behavior (characterized by the cessation of the movements except to those associated with breathing) by a blind observer. After each trial the box was carefully cleaned with a 70% alcohol solution. To prevent eventual cat smell interference, the cat-exposed group was always tested after the group exposed to the dummy cat. Seven days after cat exposure all animals were placed into the elevated plus maze facing one of the enclosed arms. The number of entries and time spent in the open and enclosed arms were recorded for 5 min to detect anxiety-like behaviors. At the end of the behavioral procedures animals were perfused and sacrificed under deep urethane anesthesia (Sigma–Aldrich, St. Louis, MO, USA, 5 ml/kg, IP). Brain tissue was removed for subsequent immunohistochemistry analysis of the following brain structures: the medial prefrontal cortex, hippocampus (dorsal/ventral), amygdaloid complex (corresponding to basolateral-BLA, medial and central nuclei) and dlPAG.

2.3.2. Experiment 2

Similar to Experiment 1 except that after EPM exposure the hippocampal formation and the prefrontal cortex were immediately dissected for posterior analyses of nitrite/nitrate (NO_x) tissue content.

2.4. Immunohistochemistry

Immediately after exposure to the EPM, all animals from experiment 1 were anesthetized and transcardially perfused with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PBS, pH 7.4). After perfusion all brains were removed and post-fixed over 2 h in a paraformaldehyde solution (4%). Then all brains were stored for at least 30 h in 30% sucrose solution for cryoprotection. Coronal sections (30 μm) were obtained in a cryostat (Criocut, Leica-Germany). Sections were first processed as previously described previously [29]. Briefly, tissue sections were washed and incubated overnight at room temperature with the primary antibody nNOS (1/1000, rabbit IgG; C-terminus; Santa Cruz Biotechnology). After incubation in the primary antiserum, the tissue sections were washed in TBS and in sequence incubated with a biotinylated goat anti-rabbit IgG (1:1000; Vector Laboratories, Burlingame, CA). Sections were then processed by the avidin–biotin immunoperoxidase method (Vectastain ABC kit; Vector Laboratories, Burlingame, CA). nNOS immunoreactivity was revealed by the addition of the chromogenic reagent diaminobenzidine (Sigma–Aldrich; into TBS, H_2O_2 0.02%) and visualized as a brown reaction product inside the neuronal cytoplasm. A minimum of 8 coronal sections of each brain region per animal were analyzed. All images were analyzed by an optical microscopy (Olympus, BX50). The prelimbic and infralimbic cortices (1-in-8 sections, 5.15–2.52 mm anterior to bregma) were analyzed together as the vmPFC. A 1-in-8 slices of the hippocampus (dorsal/ventral: 2.4–4.68 mm posterior to bregma), amygdaloid complex (corresponding to basolateral, medial and central

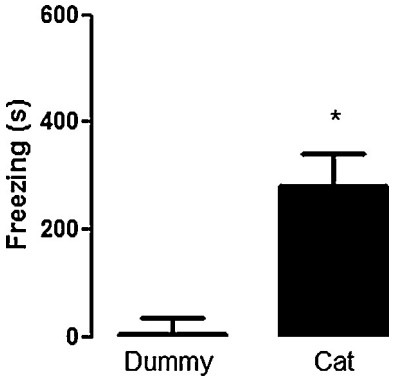

Fig. 1. Freezing behavior (time) of rats exposed to a live or a dummy cat. Columns represent mean ± SEM. * indicates significant difference between groups ($p < 0.001$, $n = 10$ and 8 animals).

nuclei: 1.92–2.52 mm posterior to bregma) and dlPAG region (6.36–7.80 mm posterior to bregma) were analyzed and the results expressed as the number of positive cells/0.1 mm^2, except for two regions: the central amygdala, which was analyzed by optical density, and the hippocampal formation, where the results were expressed as the number of cell/area.

2.5. NO_x measurements

Immediately after the EPM test, rats were decapitated and their brains immediately removed. The prefrontal cortex (2.20–4.20 mm anterior to bregma) and the whole hippocampus were quickly dissected and frozen in liquid nitrogen [26]. Before the NO_x assay the tissue was homogenized in ice-cold buffer containing 0.25 M sucrose, 0.1 mM EDTA, 50 mM K–Na phosphate buffer, pH 7.2. Homogenates were centrifuged at 15,000 × *g* for 10 min at 4 °C. The supernatant obtained by this procedure was separated from tissue pellet and immediately used or frozen and stored at −70 °C. Nitrite and nitrate were measured as described before [30]. Briefly, the assay was performed by reduction of nitrate to nitrite using a β-NADPH-dependent nitrate reductase from *Aspergillus* sp. The concentration of nitrite was measured according to the Griess reaction, with modifications [31]. Individual supernatants were incubated overnight with the enzyme β-NADPH (Sigma, USA) at 37 °C. Nitrite production was determined by mixing 40 μL of each homogenate with 40 μL of Griess reagent (1.5% sulphanilamide in 1 M HCl + 0.15% N-(1-naphthyl) ethylenediamine dihydrochloride in distilled water, v/v). After 10 min of incubation at room temperature, the absorbance at 540 nm was determined and nitrite concentrations were calculated from sodium nitrite (Sigma, USA) standard curve. The content of protein in individual homogenates was measured by the method of Bradford [32] using serum albumin as standard (Biorad, Wien, Austria). All measurements were performed in triplicate.

2.6. Statistical analysis

All data were tested for normality and homogeneity of variance. Behavioral and biochemical data were analyzed by Student's *t* test, excepted for basolateral amygdaloid nucleus, where results were analyzed by Mann–Whitney non-parametric test. Correlations were performed by Pearson's correlation test (two-tailed). The level of significance was set at $p < 0.05$.

3. Results

Fig. 1 shows that predator exposure induced a significant fear reaction reflected by an increase in freezing time ($t_{(16)} = 5.4$, $p < 0.001$). Results of the long lasting behavioral effects of predator threat can be seen in Fig. 2. Cat exposure promoted, seven days later, a decreased in the % of entries ($t_{(16)} = 2.9$, $p < 0.005$) and of time spent in the open arms ($t_{(16)} = 2.9$, $p < 0.001$) of the EPM. No changes in the number of enclosed arms entries in the EPM were found (p = ns).

The immunohistochemistry results can be observed in Figs. 3 and 4. After predator exposure, the number of nNOS positive cells neurons significantly increased in the vmPFC (Fig. 3A, $t_{(7)} = 2.6$, $p < 0.05$) and in the BLA (Fig. 4A; $U = 0$, $z = 2.45$, $p < 0.05$) but not in the other analyzed brain areas (hippocampus subfields – CA1, CA3 and dentate gyrus; amygdaloid complex, dlPAG; Fig. 4A–C).

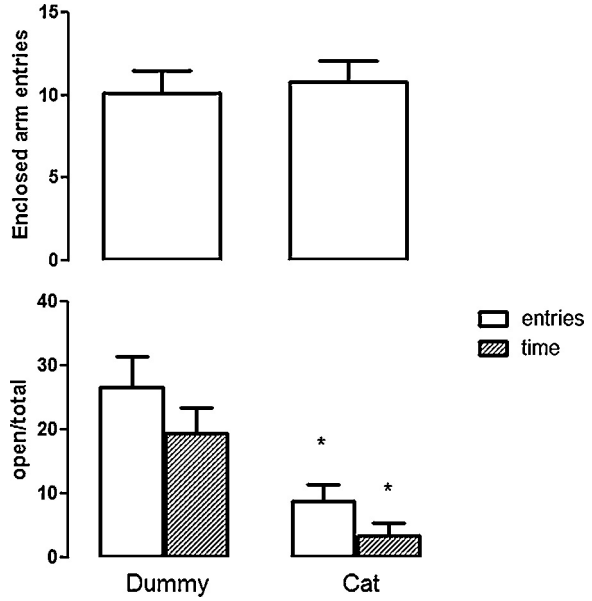

Fig. 2. Predator stress induces long lasting anxiety-like behaviors in the EMP. Animals were submitted to the EPM seven days after a 10 min predator exposure session. Columns represent mean ± SEM. In the upper panel the open columns represent the enclosed arms entries. In the lower panel open columns represent the percent of entries onto the open arms while the hatched columns represent the percent of the time spent in the open arms. * indicates significant difference from dummy exposed rats ($n = 10$ and 8, respectively).

Fig. 5 shows that predator exposure increased NO_x levels in the vmPFC ($t_{(7)} = 2.6$, $p < 0.05$; Fig. 5A) but not in the hippocampus (p = ns; Fig. 5B).

Positive correlations were found between freezing time during cat exposure and the number of nNOS positive neurons ($r = 0.70$; $p < 0.5$, $n = 9$) and NO_x levels in the prefrontal cortex ($r = 0.90$; $p < 0.01$, $n = 9$). The percentage of time spent in the open arms was also correlated with the number of nNOS neurons ($r = -0.802$, $p < 0.001$, $n = 9$).

4. Discussion

In agreement to earlier results [24,33–37], predator exposure induced a long lasting (one week) increase in anxiety response measured by the EPM. This behavioral change was associated with an increase in the number of nNOS positive neurons in the vmPFC and basolateral amygdala. NO_x levels, an indirect measure of NO production, also increased in the vmPFC.

The high liposolubility and short-life of NO is usually an obstacle to determine its physiological role in the CNS. However, the presence of positive nNOS neurons in brain areas related to the expression and modulation of defensive behaviors strongly suggests an important role for NO in stress and anxiety related responses [21]. This possibility has been supported by several studies [19,21,38–41]. For example, Kelley et al. [42] have recently demonstrated that genetic deletion of nNOS gene impairs fear conditioning, an animal model used to modelling some aspects of PTSD. Moreover, inhibition of nNOS activity or NO effects in regions such as the vmPFC, medial amygdala, premammillary dorsal nucleus and dlPAG decreases defensive responses [43–47] whereas administration of NO-donors into the dlPAG induces flight reactions [50]. Previous studies have also shown that exposure to aversive stimuli such as forced restraint can increase the number of neurons expressing nNOS in stress-related areas such as the medial amygdala, paraventricular nucleus of the hypothalamus and dlPAG [19] 24 h later. In our study, however, predator exposure failed to

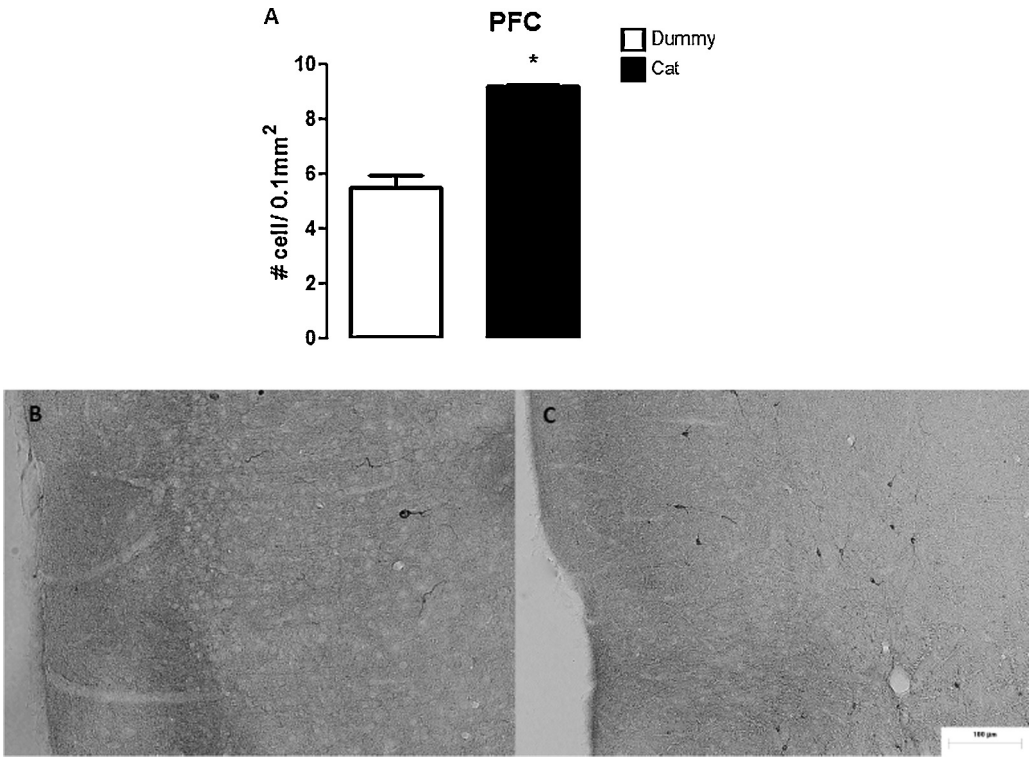

Fig. 3. In situ analysis of nNOS positive neurons in brain areas related to PTSD symptoms. (A) Prefrontal cortex; (B and C) representative images of nNOS immunohistochemistry of dummy and predator exposed rats respectively. Bar size 100 μm. Bars represent mean ± SEM. * indicates significant difference between groups ($p < 0.05$, $n = 5$ and 4 animals).

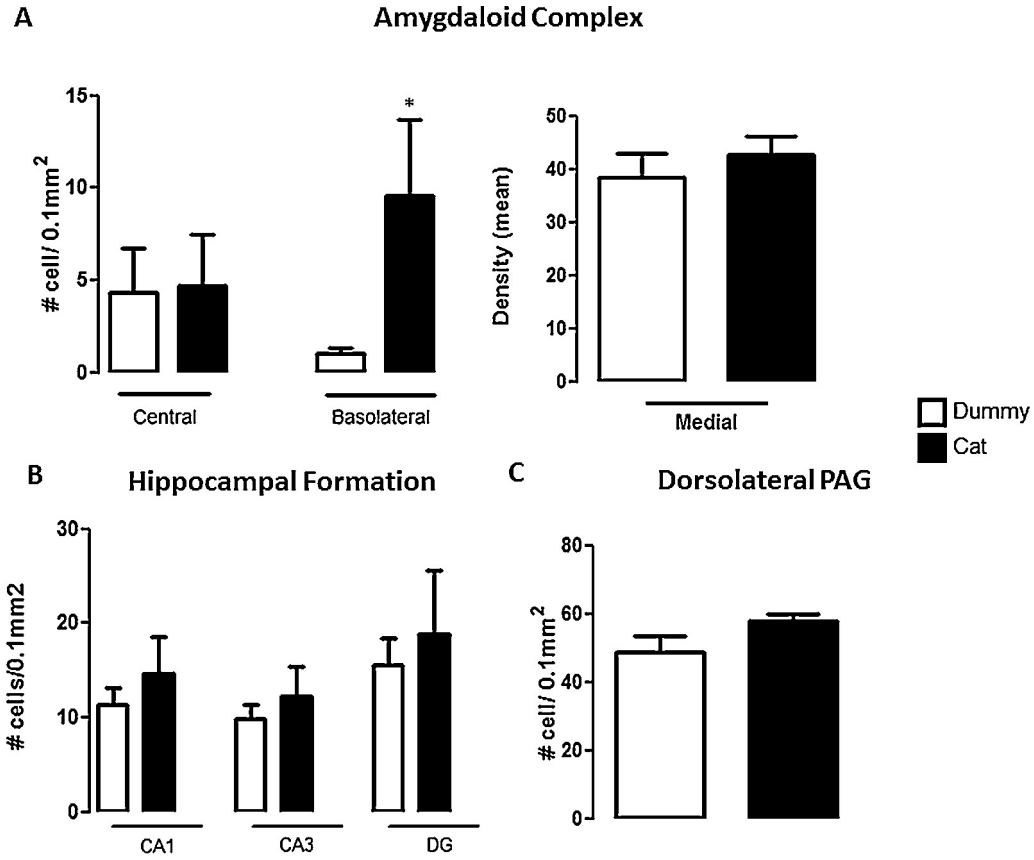

Fig. 4. nNOS positive neurons in amygdaloid complex, hippocampal formation and dlPAG of rats exposed to a predator seven days after EPM test. (A) Central, basolateral and medial nuclei of amygdala; (B) hippocampal subfields (CA1, CA3 and dentate gyrus-DG); (C) dlPAG. Further specifications, see Fig. 3.

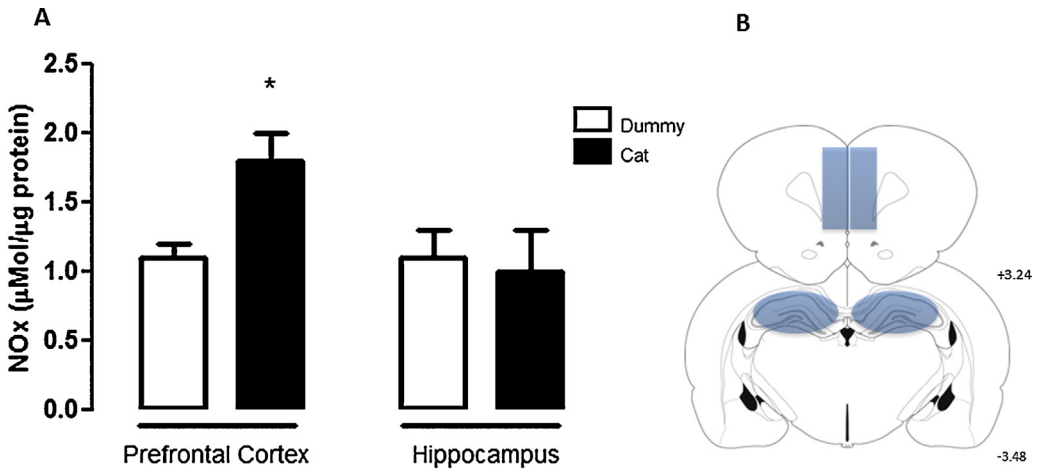

Fig. 5. NO_x levels in the brain of rats exposed to the PTSD model. (A) Prefrontal cortex (left) and Hippocampal formation (right). (B) Brain sites from where the tissue samples were extracted. The whole prefrontal cortex (PFC) and the hippocampus were dissected bilaterally. Columns represent mean ± SEM. * indicates significant difference between groups ($p < 0.05$, $n = 5$ and 4 animals).

increase nNOS expression in the hippocampal formation, central and medial nuclei of amygdala and dlPAG seven days after predator exposure. Several differences between this study and the present one, including the interval between test and tissue analysis (24 h versus one week), the nature of the stressor (restraint versus predator) and the length of stress exposure (10 min versus 2 h, in the case of predator and restraint exposure, respectively), could help to explain the present findings. Lack of statistical power to identify changes in these other regions could also be involved. However, the number of animals per group in our work was similar to those used in several immunohistochemistry studies aimed at detecting changes in neuronal activation or nNOS expression [9,26,29,43]. Moreover, this number was able to detect nNOS expression changes in the vmPFC. Therefore, even if delayed nNOS expression changes after predator exposure in other brain regions related to defensive responses cannot be ruled out, the results indicate that predator exposure induces a significant increase in NOS expression in the vmPFC and BLA. All animals were exposed to the EPM before sacrifice. Although this could be a potential limitation of the present study, it is unlikely that this exposure could have affected nNOS expression since previous studies have shown that EPM exposure is not able to acutely change the number of nNOS neurons in stress related areas [48,49]. Moreover, innate fear stimulus, such as EPM or cat exposure induces acute rapid activation of neurons containing nNOS in defensive-related brain regions such as the dlPAG and bed nucleus of the stria terminalis [48,49].

The vmPFC and amygdala, together with the hippocampus, have been implicated in the pathophysiology of PTSD by neuroimaging studies inpatients [57–59]. Moreover, recent findings have highlighted the cortical control of the amygdala in fear regulation in humans, suggesting that PTSD could result from a failure in medial PFC inhibitory control of the amygdala [60–64].

In agreement with previous studies, the total number of nNOS positive neurons in the vmPFC was smaller than other brain areas [19]. Nonetheless, NO is a freely diffusible molecule that can promptly spread and influence a large population of target cells in a 0.3–0.4 diameter (μm) spatial sphere [21]. In this area NO modulate synaptic transmission more commonly by activation of the soluble guanylate cyclase (sGC) enzyme and protein nitrosilation [51,52]. Due to the former mechanisms, NO increase glutamate release, a mechanism that could be related to the present findings. Acute inhibition of glutamatergic neurotransmission in brain regions related to defensive responses, including the vmPFC, causes anxiolytic effects [47,53–55] whereas glutamate receptor antagonists prevent the flight responses induced by NO donors in the dlPAG [56].

The PFC has been implicated in the regulation of emotional behaviors and in learning and memory processes [65,66]. Several pieces of evidence also suggested that PFC plays an important role in reactivating emotional status related to past stressful experiences [3,4,59,61,65]. Consistent with this proposal, our results demonstrate that predator threat produces long lasting-related increase in anxiety, nNOS positive neurons and NO production in the vmPFC of rats. These plastic modifications following an initial life threatening stimuli suggest that nitric oxide-mediated neurotransmission in the PFC could be directly involved in the pathogenesis of PTSD. Inhibition of nNOS in the vmPFC produces anxiolytic-like effects in fear conditioning [55], suggesting that the vmPFC-NMDA-NO pathway participates in the control of contextual fear memory expression. Also, a NOS1 variant rs6490121 is associated with changes in PFC function and grey matter density in healthy subjects [23]. More recently, a study conducted in war veterans found a positive correlation between a genetic variation of the NOS1AP gene and the risk for PTSD development. NOS1AP competes with PSD-95 for nNOS and, as a result, NMDA receptor signaling via PSD-95 and nNOS is reduced [23]. This result corroborates the hypothesis that the NMDA-NO pathway in PFC plays an important role not only in consolidation of fear/traumatic memories but also in the long lasting expression of anxiety.

5. Conclusions

Our data suggest that the long-term anxiogenic effect of severe trauma exposure could result from changes in NO-mediated neurotransmission in key areas associated with PTSD such as the vmPFC and basolateral amygdala. Although new studies aimed at testing this possibility via direct interventions with local nitrergic system are clearly needed, the present results indicate that the nitrergic system could be a new candidate for future therapeutic targets in the treatment of anxiety disorders that result from traumatic experiences.

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