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NITRIC OXIDE IN THE PRELIMBIC MEDIAL PREFRONTAL CORTEX IS INVOLVED IN THE ANXIOGENIC-LIKE EFFECT INDUCED BY ACUTE RESTRAINT STRESS IN RATS

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Abstract—Neurons containing the neuronal nitric oxide synthase (nNOS) enzyme are located in brain areas related to defensive behavior, such as the ventromedial prefrontal cortex (vMPFC). Rats exposed to a live predator (a cat) present anxiety-like behavior and an increased number of nNOS-positive neurons in this brain area one-week later. Moreover, stress-related behavioral changes in rodents can be prevented by systemic or local vMPFC nNOS inhibition. In the present study we investigated if acute restraint stress (RS)-induced delayed (one-week) anxiogenic-like effect was associated with increased nNOS expression or activity in the vMPFC. Furthermore, we also tested if local pharmacological nNOS inhibition would prevent stress-induced behavioral changes. Male Wistar rats were submitted to RS for 3 h and tested in the elevated plus maze (EPM) 24 h or 7 days later. Two hours after the EPM test, their brains were removed, processed and nNOS expression in the vMPFC was evaluated by immunohistochemistry. Another group of animals was used for measuring NO metabolites (NOx; an indirect measure of NOS activity) immediately after the EPM test, 24 h after RS. Independent groups had guide cannula implanted bilaterally into the prelimbic (PL) portion of vMPFC. Five to six days after surgery, the animals were submitted to RS and 24 h later received local administration of the nNOS inhibitor, N-propyl-L-arginine (NPLA; 0.04 nmol). They were tested in the EPM 10 min later. RS-induced anxiogenic-like effect was accompanied by increased nNOS expression in the PL ($p < 0.05$), but not in the infralimbic (IL) medial prefrontal cortex (MPFC), both 24 h and 7 days after RS. Moreover, open-arm exploration of the EPM was negatively correlated with nNOS expression ($p < 0.05$) and NOx levels ($p < 0.05$) in the PL. The anxiogenic-like effect observed 24 h after RS was prevented by NPLA ($p < 0.05$). Our results suggest that RS-induced anxiogenic-like effect might depend on increased nNOS-mediated signaling in the PL MPFC. © 2016 Published by Elsevier Ltd. on behalf of IBRO.

Key words: restraint-stress (RS), anxiety, nNOS, prelimbic medial prefrontal cortex (PL MPFC), elevated plus maze (EPM).

INTRODUCTION

Stress can induce behavioral, physiological, cognitive, and neural changes, potentially altering homeostasis and promoting vulnerability to illness (Selye, 1936, 1976; McEwen, 1998). The brain is highly susceptible to stress effects and is the crucial mediator of its behavioral and physiological effects (McEwen, 2007). Abnormal activity in the prefrontal cortex (PFC), hippocampus and amygdala are commonly observed in stress-related mental illnesses (Drevets, 2003; Shin et al., 2006), such as major depressive (Lechin et al., 1996; Hammen, 2005), generalized anxiety (Risbrough and Stein, 2006) and post-traumatic stress disorders (PTSDs) (Turner and Lloyd, 2004). The PFC is also involved with decision making, autonomic and neuroendocrine function, particularly during stressful situations (McEwen and Gianaros, 2010).

The rodent PFC is subdivided into medial (MPFC), lateral and ventro-orbital regions (Ongur and Price, 2000; Dalley et al., 2004). The ventral portion of MPFC (vMPFC) – composed by the prelimbic (PL) and infralimbic (IL) subregions- is particularly sensitivity to stress (Heidbreder and Groenewegen, 2003; Radley et al., 2006). It modulates stress-induced neuroendocrine, autonomic and behavioral changes, such as activation of the hypothalamic–pituitary–adrenal (HPA) axis; changes in breathing, heart rate, blood pressure; and anxiety-like behavior (Jinks and McGregor, 1997; Sullivan and Gratton, 2002a,b; Spencer et al., 2005; Resstel and Correa, 2006; Resstel et al., 2008b; Lisboa et al., 2010).

Glutamate increases in the vMPFC of rodents during stressful situations (Moghaddam, 1993). This neurotransmitter activates NMDA receptors increasing calcium influx, which stimulates the neuronal nitric oxide synthase (nNOS) enzyme, resulting in nitric oxide (NO) production

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Abbreviations: ANOVA, analysis of variance; AP, anteroposterior; BSA, bovine serum albumin; CNS, central nervous system; EPM, elevated plus maze; HPA, hypothalamic–pituitary–adrenal; IHC, immunohistochemistry; IL, infralimbic; MPFC, medial prefrontal cortex; NF-κB, nuclear factor κB; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NPLA, N-propyl-L-arginine; PFC, prefrontal cortex; PL, prelimbic; PTSDs, post-traumatic stress disorders; PVN, paraventricular hypothalamic nucleus; RS, restraint stress; RS, restraint stress; sGC, soluble guanylate cyclase.

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(Garthwaite et al., 1989; Guix et al., 2005; Garthwaite, 2008). NO acts as an intracellular messenger in the central nervous system (CNS), playing a modulatory role in several brain functions, such as synaptic plasticity and neuroprotection, as well as dysfunctions, such as neurotoxicity (Moncada et al., 1991; Zhang and Snyder, 1995; Prast and Philippu, 2001). Moreover, NO interferes with anxiety-related behaviors (Morato et al., 2004; Guimaraes et al., 2005; Joung et al., 2012) in brain areas that include the MPFC (Resstel et al., 2008a; Lisboa et al., 2011). The long-lasting anxiety-like behavior in rats induced by cat exposure, for example, was associated with an increase in nNOS expression and NO metabolite (NOx) levels in the MPFC (Campos et al., 2013b), suggesting that pharmacological modulation of the vMPFC nitrergic system could modify long-lasting consequences of stress.

Acute restraint stress (RS) can induces both psychological and physical effects, causing a broad range of behavioral and physiological changes, including anxiogenic-like effects (Padovan et al., 2000; Resstel et al., 2009), endocrine (Chrousos, 1998; Busnardo et al., 2010, 2013) and autonomic alterations (Resstel et al., 2009; Vianna and Carrive, 2009; Busnardo et al., 2010, 2013). In addition, RS increases the number of nNOS-expressing neurons in the hippocampus and amygdala (De Oliveira et al., 2000). The effects on RS in nNOS expression in the MPFC, however, are still unknown.

The present work, therefore, investigated the hypothesis that the anxiogenic-like effect induced by acute RS is associated with increased nitrergic-mediated neurotransmission in the vMPFC. We also tested if blocking this neurotransmission directly in the vMPFC would attenuate stress-induced anxiety-like behavior.

EXPERIMENTAL PROCEDURES

Animals

Male Wistar rats (260–300 g) originated from the Central Animal Farm of the Medical School of Ribeirão Preto, University of São Paulo (FMRP-USP), were housed in groups of five in plastic cages until the beginning of the experiments. After the stress, they were housed individually or in pairs (as described in the experimental protocol) 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 under a 12-h light/dark cycle (lights on at 6.30 a.m.). All behavioral analyses were performed during the light phase of the cycle. The Institution's Animal Ethics Committee approved the housing conditions and all experimental procedures (process number: n°006/2013).

Drugs

The selective nNOS inhibitor, N-propyl-L-arginine (NPLA, 0.04 nmol; Tocris, USA), was dissolved in saline (NaCl 0.9%). The NPLA dose was based on previous studies (Zhang et al., 1997; Resstel et al., 2008a). Tribromoethanol (2.5%, 10 ml/kg, i.p.; Sigma–Aldrich, USA) and Chloral hydrate (5.0%, 10 ml/kg, i.p.; Sigma–Aldrich, USA) were dissolved in distilled water.

Methods

RS procedure. Animals were submitted to RS in metallic tubes (6.3 × 19.3 cm), with an adjustable roof ventilated by holes, for 3 h at room temperature. Considering that post-stress housing could interfere on RS delayed behavioral changes (Andrade and Guimaraes, 2003), immediately after stress rats were housed individually or in pairs according to the experimental protocol, until the end of the behavioral test.

Elevated plus maze (EPM) test. Twenty-four hours or 7 days after RS the animals were tested in the EPM. The apparatus had two opposite open arms (50 × 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 one 40-W incandescent light placed 3 m away from 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 (Pellow et al., 1985; Carobrez and Bertoglio, 2005). The Any maze software (version 4.5, Stoelting, Wood Dale, USA) was employed for behavioral analysis. The animal image captured by a video camera placed above the apparatus during 5 min is processed by this software, which calculates the percentage of entries (%entries) and time (%time) spent in the open arms and the number of enclosed arms entries. After each trial, the maze was cleaned with a solution of ethanol 70%.

Immunohistochemistry (IHC). Two hours after exposure to the EPM the animals were anesthetized with chloral hydrate, the chest was surgically opened, the descending aorta occluded, the right atrium punctured and the brain 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%) and stored for at least 48 h in 30% sucrose solution for cryoprotection. Coronal brain sections (40 μm) were cut in a cryostat (Criocut, Leica, Germany), based on the Paxinos and Watson atlas (2006) (Paxinos and Watson, 2006). For the PL MPFC region, sections 6–12 from the atlas (5.16–2.76 mm anterior to Bregma) were used, whereas for the IL region, sections 9–12 (3.76–2.76 mm anterior to the Bregma) were employed.

The sections were processed as previously described (Aguiar and Guimaraes, 2009). Briefly, tissue sections were washed and incubated overnight at 4 °C with the primary antibody nNOS (R20 – sc-648, 1:1000, rabbit IgG; C-terminus; Santa Cruz Biotechnology). After incubation in the primary antiserum, the tissue sections were washed

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