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Research report

Adenosine A_1 receptor activation modulates N-methyl-D-aspartate (NMDA) preconditioning phenotype in the brain

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HIGHLIGHTS

- NMDA preconditioning slightly increases A_1R binding affinity in the hippocampus.
- NMDA preconditioning does not alter A_1R levels in the hippocampus.
- NMDA preconditioning evokes antinociception *in vivo*.
- NMDA preconditioning increases glutamate uptake into hippocampal slices.
- A_1R activation precludes these NMDA-mediated functional responses.

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ABSTRACT

N-methyl-D-aspartate (NMDA) preconditioning is induced by subtoxic doses of NMDA and it promotes a transient state of resistance against subsequent lethal insults. Interestingly, this mechanism of neuroprotection depends on adenosine A_1 receptors (A_1R), since blockade of A_1R precludes this phenomenon. In this study we evaluated the consequences of NMDA preconditioning on the hippocampal A_1R biology (*i.e.* expression, binding properties and functionality). Accordingly, we measured A_1R expression in NMDA preconditioned mice (75 mg/kg, i.p.; 24 h) and showed that neither the total amount of receptor, nor the A_1R levels in the synaptic fraction was altered. In addition, the A_1R binding affinity to the antagonist [^{3}H] DPCPX was slightly increased in total membrane extracts of hippocampus from preconditioned mice. Next, we evaluated the impact of NMDA preconditioning on A_1R functioning by measuring the A_1R-mediated regulation of glutamate uptake into hippocampal slices and on behavioral responses in the open field and hot plate tests. NMDA preconditioning increased glutamate uptake into hippocampal slices without altering the expression of glutamate transporter GLT-1. Interestingly, NMDA preconditioning also induced antinociception in the hot plate test and both effects were reversed by post-activation of A_1R with the agonist CCPA (0.2 mg/kg, i.p.). NMDA preconditioning or A_1R modulation did not alter locomotor activity in the open field. Overall, the results described herein provide new evidence that post-activation of A_1R modulates NMDA preconditioning-mediated responses, pointing to the importance of the cross-talk between glutamatergic and adenosinergic systems to neuroprotection.

Abbreviations: CNS, central nervous system; NMDA, N-methyl-D-aspartate; A_1R, A_1 receptors; PKA, protein kinase A; PI3K, phosphatidylinositol-3 kinase; CPA, cyclo-pentyl-adenosine; CCPA, 2-chloro-N6-cyclopentyl-adenosine; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; KRB, Krebs-Ringer bicarbonate buffer; MTT, 3-(4,5-dimethylthiazol-2-yl)-diphenyltetrazolium bromide; GLT-1, glutamate transporter-1; HBSS, Hank's balanced salt solution.

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1. Introduction

Preconditioning was originally described in a seminal study proposing that multiple brief ischemic episodes protect the heart from a subsequent sustained ischemic insult [1]. Interestingly, this preconditioning phenomenon is not limited to the heart tissue but also extended to the central nervous system (CNS) and protection may be achieved, not only against ischemia, but also to different brain insults. Preconditioning can be defined as reaching a state of protection in a given tissue or organism through exposure to sub-lethal insults. Preconditioned individuals or tissues acquire a certain tolerance to a subsequent lethal insult, decreasing cell death resultant from injury [1,2]. This brain tolerance to lethal injury may be achieved after chemical, electrical or anoxic stimuli [3–6].

Molecular mechanisms underlying preconditioning are not completely elucidated, but N-methyl-D-aspartate (NMDA) glutamate receptors, nitric oxide synthase, cytokines, oxidative stress and mitochondrial bioenergetics modulation and suppression of the innate immune system are some of the elements that have a pivotal role in such process [7–10]. Particularly, NMDA and glutamate may act as chemical preconditioning agents in hippocampal slices [11], in cultured cells [12] and *in vivo* models of excitotoxicity [13]. Subtoxic doses of NMDA (*e.g.*, 75 mg/kg, i.p.) yield preconditioning against chemically-induced seizures [14,13] or trauma brain injury *in vivo* [15]. Administration of NMDA receptor antagonists, such as MK-801 or ketamine, prevents NMDA-induced preconditioning, confirming the dependence on NMDA receptors activation to achievement of a preconditioned state [3–6]. Regarding intracellular signaling pathways, we have shown that inhibition of protein kinase A (PKA) or phosphatidylinositol-3 kinase (PI3K) pathways activation abolishes NMDA preconditioning against the neurotoxicity and seizures induced by quinolinic acid, an NMDA receptor agonist [16]. Moreover, a proteomic analysis of the hippocampus of NMDA preconditioned mice, showed a differential expression of proteins involved in translation and in protein processing, in the energy homeostasis maintenance and modulation of glutamatergic transmission [17].

The dependence of NMDA preconditioning on adenosine A_1 receptors (A_1R) activation has already been reported *in vivo* and *in vitro*. For instance, the blockade of A_1R with the selective antagonist 8-cyclopentyl-1,3-dimethylxanthine was able to abolish the protective effect evoked by NMDA preconditioning against quinolinic acid-induced seizures in mice [13] or in cerebellar granule cells challenged with glutamate [18]. The cellular protection achieved by chemical preconditioning with NMDA occurs by increasing extracellular adenosine levels [19–21]. In its turn, adenosine modulates glutamatergic excitotoxicity caused by overstimulation of NMDA receptors [22]. Glutamatergic transmission in the hippocampus is also part of a nociceptive pathway, since blockade of NMDA receptors in the hippocampus prevent the induction of chemical pain by formalin [23]. Since activation of A_1R reduces glutamatergic transmission, modulation of nociception *via* A_1R inhibition of glutamate release may be a viable mechanism of analgesia [24]. In the present study, we aimed to understand potential NMDA preconditioning-mediated changes in hippocampal A_1R expression and binding properties that may underlie the neuroprotective mechanism of this phenomenon. Moreover, a putative effect of A_1R activation after NMDA preconditioning on its effects on glutamate uptake into hippocampal slices and in behavioral responses in the open field and hot plate tests was assessed.

2. Material and methods

2.1. Animals

Male adult Swiss albino mice (30–40 g) were maintained on a 12 h light/12 h dark schedule (lights on at 7:00 a.m.) at 25 °C. Mice were housed in plastic cages with food and water *ad libitum*. All manipulations were carried out between 9:00 and 16:00 h. All experimental procedures involving the animals were performed in accordance with National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23), and were designed to minimize suffering and limit the number of animals used. The experiments were performed after approval of the protocol by the local Institutional Ethics Committee for Animal Research (CEUA/UFSC PP0549).

2.2. Drugs

N-methyl-D-aspartate (NMDA), cyclo-pentyl-adenosine (CPA) and 2-chloro-N6-cyclopentyl-adenosine (CCPA) were from Sigma–Aldrich Chemical Co. (MO, USA). [^{3}H] 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, 120.0 Ci/mmol) was from PerkinElmer (MA, USA) and [^{3}H] L-glutamate (31.0 Ci/mmol) was from GE-Healthcare (UK).

2.3. NMDA preconditioning and mice treatment

NMDA was dissolved in 0.9% NaCl (saline) solution and adjusted to pH 7.4 with NaOH 1 mEq/mol. Animals were pretreated with NMDA at a subtoxic and non-convulsive dose (75 mg/kg; i.p.) or vehicle (saline 0.9%; i.p.). Animals were observed for 30 min immediately after the administration of NMDA, for the occurrence of any behavioral alteration [13]. Preconditioned mice were all evaluated 24 h after NMDA treatment. When administered *in vivo*, the adenosine A_1R agonist, CCPA, was injected 30 min before behavioral evaluations or glutamate uptake assay. CCPA was dissolved in saline with 0.4% DMSO. The dose of CCPA (0.2 mg/kg, i.p.) was chosen based on previous studies [25]. NMDA and CCPA were administered by intraperitoneal (i.p.) route in a constant volume of 10 mL/kg body weight.

2.4. Hippocampal cell viability

Mice were pretreated with NMDA (75 mg/kg, i.p.) or vehicle (saline 0.9%, i.p.). After 24 h, animals were killed by decapitation and hippocampi were rapidly removed. During dissection hippocampi were maintained in an ice-cold Krebs-Ringer bicarbonate buffer (KRB) with the following compositions (in mM): 122 NaCl, 3 KCl, 1.2 $MgSO_4$, 1.3 $CaCl_2$, 0.4 KH_2PO_4, 25 $NaHCO_3$, and 10 D-glucose. The buffer was bubbled with 95% O_2–5% CO_2 up to pH 7.4 [26]. Slices (0.4 mm) were prepared using a McIlwain Tissue Chopper and separated in KRB at 4 °C. Immediately after sectioning, slices were transferred to vials with fresh KRB, bubbled with 95% O_2/5% CO_2 for 30 min at 35 °C to recover from slicing trauma, before starting the experiments (equilibration period). Cell viability was determined through the ability of cells to reduce 3-(4,5-dimethylthiazol-2-yl)-diphenyltetrazolium bromide (MTT; Sigma). Hippocampal slices were then incubated with MTT (0.5 mg/mL) in KRB for 30 min at 37 °C [27]. The tetrazolium ring of MTT can be cleaved through reduction by dehydrogenases into viable cells in order to produce a precipitated formazan. The formazan produced was solubilized by adding 200 μL of dimethyl sulfoxide (DMSO), resulting in a colored compound from which optical density was measured in an ELISA reader (550 nm).