Neuropharmacology 89 (2015) 274–281

Contents lists available at ScienceDirect

Neuropharmacology

journal homepage: www.elsevier.com/locate/neuropharm

Glycine transporters type 1 inhibitor promotes brain preconditioning against NMDA-induced excitotoxicity

Mauro Cunha Xavier Pinto [a, e, *], Isabel Vieira de Assis Lima [b], Flávia Lage Pessoa da Costa [a, e], Daniela Valadão Rosa [a], Vânia Aparecida Mendes-Goulart [c], Rodrigo Ribeiro Resende [c], Marco Aurélio Romano-Silva [a], Antônio Carlos Pinheiro de Oliveira [b], Marcus Vinícius Gomez [d], Renato Santiago Gomez [e]

[a] INCT de Medicina Molecular, Faculdade de Medicina, Universidade Federal de Minas Gerais, Av. Alfredo Balena 190, 30130-100, Belo Horizonte, MG, Brazil
[b] Departamento de Farmacologia, Instituto de Ciência Biológicas, Universidade Federal de Minas Gerais, Av. Antônio Carlos, 6627, 31270-901, Belo Horizonte, MG, Brazil
[c] Departamento de Bioquímica e Imunologia, Instituto de Ciência Biológicas, Universidade Federal de Minas Gerais, Av. Antônio Carlos, 6627, 31270-901, Belo Horizonte, MG, Brazil
[d] Instituto de Ensino e Pesquisa da Santa Casa de Belo Horizonte, R. Domingos Vieira, 590, Belo Horizonte, MG, Brazil
[e] Departamento de Cirurgia, Faculdade de Medicina, Universidade Federal de Minas Gerais, Av. Alfredo Balena, 190, 30130-100, Belo Horizonte, MG, Brazil

ARTICLE INFO

Article history:
Received 20 May 2014
Received in revised form
7 September 2014
Accepted 1 October 2014
Available online 13 October 2014

Keywords:
Brain preconditioning
Neuroprotection
Neurotransmission
Glycine transporter type 1

ABSTRACT

Brain preconditioning is a protective mechanism, which can be activated by sub-lethal stimulation of the NMDA receptors (NMDAR) and be used to achieve neuroprotection against stroke and neurodegenerative diseases models. Inhibitors of glycine transporters type 1 modulate glutamatergic neurotransmission through NMDAR, suggesting an alternative therapeutic strategy of brain preconditioning. The aim of this work was to evaluate the effects of brain preconditioning induced by NFPS, a GlyT1 inhibitor, against NMDA-induced excitotoxicity in mice hippocampus, as well as to study its neurochemical mechanisms. C57BL/6 mice (male, 10-weeks-old) were preconditioned by intraperitoneal injection of NFPS at doses of 1.25, 2.5 or 5.0 mg/kg, 24 h before intrahippocampal injection of NMDA. Neuronal death was evaluated by fluoro jade C staining and neurochemical parameters were evaluated by gas chromatography–mass spectrometry, scintillation spectrometry and western blot. We observed that NFPS preconditioning reduced neuronal death in CA1 region of hippocampus submitted to NMDA-induced excitotoxicity. The amino acids (glycine and glutamate) uptake and content were increased in hippocampus of animals treated with NFPS 5.0 mg/kg, which were associated to an increased expression of type-2 glycine transporter (GlyT2) and glutamate transporters (EAAT1, EAAT2 and EAAT3). The expression of GlyT1 was reduced in animals treated with NFPS. Interestingly, the preconditioning reduced expression of GluN2B subunits of NMDAR, whereas did not change the expression of GluN1 or GluN2A in all tested doses. Our study suggests that NFPS preconditioning induces resistance against excitotoxicity, which is associated with neurochemical changes and reduction of GluN2B-containing NMDAR expression.

1. Introduction

The N-methyl-D-aspartate receptor (NMDAR) are ionotropic receptors activated by glutamate and its co-agonists, which allows the influx of Ca^{2+} into the cells (Stocca and Vicini, 1998; Chen et al., 2003; Papouin et al., 2012). These receptors are differently regulated by different co-agonists (D-serine or glycine) and are composed by two types of subunits: GluN1 subunit is binding site for co-agonists and GluN2 subunit is the binding site for glutamate and postsynaptic density protein-95 (PSD-95) (Massey et al., 2004;

Abbreviations: ACSF, artificial cerebrospinal fluid; EAAT, excitatory amino-acid transporter; GC–MS, gas chromatography – mass spectrometry; GlyT1, glycine transporters type 1; GlyT2, glycine transporters type 2; NFPS, *N*-[3-([1,1-Biphenyl]-4-yloxy)-3-(4-fluorophenyl)propyl]-*N*-methylglycine; NMDAR, N-methyl-D-aspartate receptor; NO, Nitric Oxide; OGD, oxygen-glucose deprivation; PKC, Protein kinase C; PSD, postsynaptic density protein; ROS, reactive oxygen species.

* Corresponding author. Faculdade de Medicina, Universidade Federal de Minas Gerais, Av. Professor Alfredo Balena, 190. Sala 114, Bairro Santa Efigênia, CEP: 30.130-100, Belo Horizonte, Minas Gerais, Brazil. Tel.: +55 (0)31 3409 9753.
E-mail addresses: mauroxavier@ufmg.br, pintomcx@gmail.com (M. Cunha Xavier Pinto).

http://dx.doi.org/10.1016/j.neuropharm.2014.10.003
0028-3908/

Zhao and Constantine-Paton, 2007; Papouin et al., 2012). In forebrain, two types of GluN2 subunits are especially relevant, GluN2A and GluN2B, which present different function and distribution in neurons (Choo et al., 2012; Papouin et al., 2012). GluN2B is expressed at both synaptic and extrasynaptic sites, whereas GluN2A is mainly expressed at the synapse (Lavezzari et al., 2004; Papouin et al., 2012). The synaptic NMDAR are important for the long-term potentiation (LTP), which requires activation of GluN2A-containing NMDAR, whereas the long-term depression (LTD) requires the activation of GluN2B-containing NMDAR (Massey et al., 2004; Zhao and Constantine-Paton, 2007). On the other hand, activation of GluN2B-containing NMDAR have been more associated with a death signal than GluN2A-containing NMDAR, inducing Ca^{2+} accumulation, mitochondrial swelling and neuronal degeneration during excitotoxicity (Choo et al., 2012; Martel et al., 2012).

The control of NMDAR activation is crucial for neuronal function and viability. High doses of NMDA lead to apoptosis and cell death through excitotoxicity, oxidative and nitrosative stress, however, neuroprotection can be induced by low doses of NMDA (Wang et al., 1999; Wang et al., 2000; Rujescu et al., 2006). This process, which is named NMDA preconditioning, induces neuroprotection against several excitotoxic injuries, including those produced by ischemia, quinolinic acid or kainate (Ogita et al., 2003; Boeck et al., 2004; Miao et al., 2005; Soriano et al., 2006). Despite the low clinical applicability of NMDA preconditioning due the risk of excitotoxicity, these evidences indicate that compounds that potentiate the activation of NMDA receptors have potential to induce brain preconditioning and to induce tolerance against excitotoxicity (Lim et al., 2004).

Glycine transporter type 1 (GlyT1) inhibitors are capable to indirectly potentiate the NMDAR function. GlyT1 protein is associated to glutamatergic synapses where its main function is to control the level of glycine (Zafra et al., 1995a; Zafra et al., 1995b; Cubelos et al., 2005; Jiménez et al., 2011; Vargas-Medrano et al., 2011). The inhibition of GlyT1 promotes an increase on glycine levels in synaptic terminals, which potentiates the NMDAR activation (Bergeron et al., 1998; Chen et al., 2003; Kinney et al., 2003; Lim et al., 2004; Zhang et al., 2009). Due to this pharmacological profile, GlyT1 inhibitors have presented activity in models of schizophrenia, epilepsy and cognitive defects, as well as they reveal a potential to induce brain preconditioning (Hashimoto et al., 2008; Kalinichev et al., 2010; Socala et al., 2010; Pinto et al., 2012, 2014b).

The brain preconditioning induced by sarcosine, a GlyT1 inhibitor, promotes ischemic tolerance in *in vitro* and *in vivo* models of brain ischemia (Pinto et al., 2012), (Pinto et al., 2014b). This effect was related to reduction of excitotoxic mediators during the ischemia and it was related to reduction of GluN2B-containing NMDAR (Pinto et al., 2012, 2014b). Besides that, whether the neuroprotective effect observed is related to the blocking of GlyT1 or it is an intrinsic effect of the sarcosine molecule it is not yet possible to state, since sarcosine also present a co-agonist activity of NMDAR (Zhang et al., 2009). In this work, we evaluated the brain preconditioning induced by NPFS, a strong and selective GlyT1 inhibitor against NMDA-induced excitotoxicity, and we study the impact of this treatment on glycinergic and glutamatergic neurotransmission.

2. Materials and methods

2.1. Animals and treatment

The experiments were performed using male C57Bl/6, weighing 25–30 g with 10–12 weeks-old. The animals were provided by the Animal Care Facilities (CEBIO) from Institute of Biological Sciences – Federal University of Minas Gerais (ICB-UFMG), Belo Horizonte-MG, Brazil. The animals were maintained on a 12-h dark–light cycle, at 25 °C controlled room, with free access to water and food. The experimental protocols were approved by Ethics Committee for Animal Experimentation from UFMG (Protocol No. 182/13). As such, all efforts were made to minimize animal suffering, to reduce the number of animals used, and to consider alternatives to *in vivo* techniques.

N-[3-([1,1-Biphenyl]-4-yloxy)-3-(4-fluorophenyl)propyl]-*N*-methylglycine – NFPS (Tocris Bioscience, Bristol, UK) was dissolved in physiological saline with 10% of 2-Hydroxypropyl-β-cyclodextrin - HP-β-CD (Sigma–Aldrich, St. Louis, MO, USA). Different doses of NFPS (1.25 mg/kg, 2.5 mg/kg and 5.0 mg/kg) were administered by intraperitoneal (i.p.) injection and control animals received vehicle injections. The procedures and analysis were performed 24 h after the drug injection.

2.2. Lesion surgery and Fluoro-Jade staining

Hippocampal lesion was induced as described by Khosravani et al., 2008 (Khosravani et al., 2008). Mice were intraperitoneally anesthetized with a mixture of ketamine (85 mg/kg) and xylazine (8.5 mg/kg), placed in stereotaxic apparatus (Insight, São Paulo-SP, Brazil) and guide cannulas were implanted using the following coordinates: 1.9 mm caudal to bregma, 1.5 mm lateral to midline, and 1.8 mm ventral from the bone surface. After 5 days of recovery, the animals received the treatment with NFPS or the control vehicle. Twenty-four hours after the brain preconditioning, the mice received hippocampal injections of 1 μL containing 40 nmol NMDA (Sigma–Aldrich, St. Louis, MO, USA) through the guide cannulas. NMDA was injected over 3 min, and the needle was left in place for an additional 2 min to minimize the leakage. Twenty-four hours after NMDA injection, mice were deeply anesthetized with ketamine/xylazine and transcardially perfused with PBS followed by 4% PFA in PBS. The brains were removed, placed in 4% PFA overnight at 4 °C, and dehydrated in 30% sucrose/PBS for 2 d. After dehydration, brain samples were preserved in −80 °C.

The frozen brain were cut in 30 μm coronal sections by cryostat, mounted on the slide glass, dried, and immersed in a basic alcohol solution (1% sodium hydroxide in 80% ethanol) for 5 min, followed by 2 min in 70% ethanol and 2 min in distilled water. After this step, slides were transferred to a solution of 0.06% potassium permanganate for 20 min. Slides were rinsed for 2 min in distilled water and were then transferred to the Fluoro Jade C (FJC) (Millipore Corporation, Billerica, MA, USA) staining solution for 20 min. The 0.0001% working solution of FJC was prepared by adding 1 mL of stock FJC solution (0.01%) to 99 mL of 0.1% acetic acid in distilled water. After staining, sections were rinsed two times (1 min) in distilled water. After be dried, the slides were coverslipped with DPX Mountant for histology (Sigma–Aldrich, St. Louis, MO, USA).

Images were collected by a blinded observer using an Imager M2 microscope (Zeiss-Germany) outfitted with a fluorescent source (original magnification ×40). To improve the image analysis, images were processed using the Image J software. The cells were defined as connected pixels that were above the threshold calculated using the image histogram. Regions in the CA1 area of hippocampus were selected from the threshold images and to calculate the percentage of threshold area in the image, which reflects the number of degenerated cells. It was analyzed both hippocampi of three slice sections of five animals per group.

2.3. [^{3}H]Glutamate and [^{3}H]Glycine uptake assays

Uptake of [3,4-^{3}H]-glutamic acid and [2–^{3}H]-glycine were assessed as previously described (Brown et al., 2003) with some modifications. Initially, it was manufactured an artificial cerebrospinal fluid (ACSF) with Sigma–Aldrich chemicals (Sigma–Aldrich, St. Louis, MO, USA) containing: 1.2 mmol/L KH_2PO_4, 2.0 mmol/L KCl, 2.0 mmol/L $MgSO_4$, 2.0 mmol/L $CaCl_2$, 26.0 mmol/L $NaHCO_3$, 127.0 mmol/L NaCl, 10.0 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 10.0 mmol/L glucose, bubbled with carbogenic mixture (95% v/v O_2 and 5% v/v CO_2). The final pH was adjusted to 7.4 with NaOH.

Twenty-four hours after the treatment with NFPS and control vehicle, the animals were euthanized; the brains were carefully removed and put in aerated and cooled ACSF. The hippocampus was dissected and hippocampal slices of 400 μm were cut with a McIlwain Tissue Chopper (Brinkman, Westbury, NY, USA). The hippocampal slices were incubated for 30 min in oxygenated ACSF at 37 °C for recovering. After that, the individual chambers containing the hippocampal slices were incubated with ACSF containing 1 μmol/L of [3,4-^{3}H]-Glutamic Acid (1.0 μCi/mmol) or [2-^{3}H]-Glycine (1.0 μCi/mmol) for 5 min at 37 °C. The hippocampal slices were collected and resuspended in 500 μL of lysis buffer solution and disrupted by probe sonication. Aliquots (100 μL) of the homogenate were added to 1 mL of scintillation cocktail. Tritium content of the samples was measured by liquid scintillation spectroscopy (Tri-Carb 2910TR, Perkin Elmer, Waltham, MA, USA).

2.4. Sample preparation procedures

For neurochemical analysis of amino acids content and protein expression, a lysis buffer solution was prepared with Sigma FAST Protease Inhibitor cocktail Tablet (Sigma–Aldrich, St. Louis, MO, USA), which contains 2.0 mmol/L 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), 1.0 mmol/L Phosphoramidon, 130.0 mmol/L Bestatin, 14.0 mmol/L E-64, 1.0 mmol/L Leupeptin, 0.2 mmol/L Aprotinin, 10.0 mmol/L Pepstatin A. 50.0 mmol/L sodium fluoride and 1 mmol/L sodium orthovanadate were added and the final pH adjusted to 7.2. The hippocampi were separated and placed in 1.5 mL tubes. The samples were homogenized in 500 μL of lysis buffer solution for 30 s. After homogenization, the samples were