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CAFFEINE ALTERS GLUTAMATE–ASPARTATE TRANSPORTER FUNCTION AND EXPRESSION IN RAT RETINA

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Abstract—L-Glutamate and L-aspartate are the main excitatory amino acids (EAAs) in the Central Nervous System (CNS) and their uptake regulation is critical for the maintenance of the excitatory balance. Excitatory amino acid transporters (EAATs) are widely distributed among central neurons and glial cells. GLAST and GLT1 are expressed in glial cells, whereas excitatory amino acid transporter 3/excitatory amino acid carrier 1 (EAAT3/EAAC1) is neuronal. Different signaling pathways regulate glutamate uptake by modifying the activity and expression of EAATs. In the present work we show that immature postnatal day 3 (PN3) rat retinas challenged by L-glutamate release [^{3}H]-D-Aspartate linked to the reverse transport, with participation of NMDA, but not of non-NMDA receptors. The amount of [^{3}H]-D-Aspartate released by L-glutamate is reduced during retinal development. Moreover, immature retinae at PN3 and PN7, but not PN14, exposed to a single dose of 200 or 500 μM caffeine or the selective A2A receptor (A2AR) antagonist 100 nM ZM241385 decreased [^{3}H]-D-Aspartate uptake. Caffeine also selectively increased total expression of EAAT3 at PN7 and its expression in membrane fractions. However, both EAAT1 and EAAT2 were reduced after caffeine treatment in P2 fraction. Addition of 100 nM DPCPX, an A1 receptor (A1R) antagonist, had no effect on the [^{3}H]-D-Aspartate uptake. [^{3}H]-D-Aspartate release was dependent on both extracellular sodium and DL-TBOA, but not calcium, implying a transporter-mediated mechanism. Our results suggest that in the developing rat retina caffeine modulates [^{3}H]-D-Aspartate uptake by blocking adenosine A2AR.

Key words: adenosine, A2A receptor, glutamate transport, NMDA receptors, retina.

INTRODUCTION

The amino acid L-glutamate is considered a major mediator of excitatory signaling in the central nervous system (CNS), including the retina from different species (Martins and Pearson, 2008; de Souza et al., 2012). Glutamatergic activity is mediated by a variety of ionotropic and metabotropic receptors in the CNS. Ionotropic N-methyl-D-aspartate (NMDA) receptors are involved in many events during development, including dendritic spine formation, maintenance and remodeling (McKinney, 2010). Prolonged activation of ionotropic glutamate receptors can lead to excitotoxicity (Ferreira et al., 1996). Therefore its extracellular levels must be highly regulated in order to avoid neuronal injury (Ishikawa, 2013).

Glutamate-aspartate transporters or excitatory amino acid transporters (EAATs) are essential for the maintenance of glutamate homeostasis. EAATs are widely distributed in central neurons and glial cells (Danbolt, 2001; Martinez-Lozada et al., 2011). They are driven by Na^+ and K^+ gradients (Jiang and Amara, 2011) and five different 'high-affinity' glutamate transporters have been cloned: GLAST (EAAT1) (Storck et al., 1992), GLT-1 (EAAT2) (Pines et al., 1992), EAAC1

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Abbreviations: cAMP, cyclic adenosine monophosphate; CNS, central nervous system; DPCPX, 8-Cyclopentyl-1,3-dipropylxanthine; EAA, excitatory amino acid; EAAT, excitatory amino acid transporter; NMDA, N-methyl-D-aspartate; TTBS, Tween 20 Tris-Buffered Saline.

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(EAAT3) (Kanai and Hediger, 1992), EAAT4 (Fairman et al., 1995) and EAAT5 (Arriza et al., 1997).

Adenosine is an important neuromodulator present in the CNS (Paes-De-Carvalho, 2002; Sebastiao and Ribeiro, 2015). Adenosine receptors and glutamate transporters are present very early in the vertebrate retina, acting on synaptic activity and cellular differentiation in this tissue (Kubrusly et al., 1998; Paes-De-Carvalho, 2002; Stutz et al., 2011). It has been identified serine residues that can be phosphorylated by protein Kinase A (PKA) on all EAAT subtypes (Gegelashvili and Schousboe, 1997), changing their activity and expression on cell surface (Garcia-Tardon et al., 2012).

Four adenosine receptor (ARs) subtypes have been cloned, namely A1, A2A, A2B and A3 receptors. The subtypes A1R and A3R are usually coupled to inhibitory G-proteins (Gi and Go) while A2AR and A2BR are coupled to stimulatory G-proteins (Gs). A1R activation inhibits adenylyl cyclase (AC), while A2AR activates this enzyme (Ribeiro et al., 2002), leading to a decrease and increase in cyclic adenosine monophosphate (cAMP) levels, respectively. We have recently shown that caffeine, a non-selective adenosine receptor antagonist, potentiates D-aspartate-stimulated GABA release in the chick retina (Ferreira et al., 2014). However, there is little information concerning the participation of adenosine receptors in glutamate homeostasis. More specifically, whether adenosine modulators, as caffeine, could influence glutamate transporters activity.

Therefore, we aimed to investigate the activity of EAATs during post natal development of rat retinas and how this transport can be modulated by a single caffeine exposure. Here we show that EAA uptake and release are developmentally changed and that caffeine modulates [^{3}H]-Aspartate transport by binding to A2AR in "ex-vivo" retinas.

EXPERIMENTAL PROCEDURES

Animals

Lister hooded rats were obtained and maintained at the animal care facility at the Instituto de Biologia, Universidade Federal Fluminense. Postnatal day 3 (PN3; day of birth considered PN0), PN7 and PN14 rats were used in this work. Retina samples were collected from 4 to 6 rats at each age. The rats were decapitated and the retinas were rapidly dissected, isolated and collected in cold phosphate-buffered saline. The research was conducted in accordance with the guidelines of the Brazilian Society for Neuroscience and Behavior (SBNeC), and with the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals. We confirm that all animal use received a formal approval from the Institutional Animal Care and Use Committee (Protocol # 00196/10). Efforts were made to reduce the number of used animals and their suffering. The following numbers of animals were used for each figure: 1A = 26, 1B = 12, 1C = 23, 2A = 27, 2B = 21, 3A = 36, 3B = 20, 4A = 18, 4B = 18, 4C = 31, 4D = 17, 4E = 18, 5A = 23 and 5B = 16. A total number of 306 animals were used for all experiments.

Material

Drugs purchased from Sigma–Aldrich (MA, EUA) were: 1,3,7Trimethylxanthine (caffeine), 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX) and 4-(-2-[7-amino-2-[2-furyl][1,2,4]triazolo[2,3-a][1,3,5]triazin-5-yl-amino]ethyl)phenol (ZM241385); [^{3}H]-D-Aspartate was purchased from PerkinElmer, Inc (MA EUA) with a specific activity of 12.2 Ci/mol. All other chemicals were of the highest purity obtainable from regular commercial sources.

[^{3}H]-D-Aspartate uptake

Rat retinas were incubated with caffeine in 1 mL of MEM (Minimum Essential Medium) at the described times and concentrations and then with 1 μCi [^{3}H]-D-Aspartate (1.2×10^{-6} M) buffered at pH 7.4 with 20 mM HEPES added at 37 °C or 4 °C (to block aspartate uptake). In some experiments, other drugs of interest (DPCPX 100 nM, ZM241385 100 nM, SCH58261 100 nM or 500 nM, DL-TBOA 0.1, 1, 10 or 100 μM or caffeine 200 or 500 μM) were used. The medium was then discarded and the tissue was rinsed six times with 2 mL of cold Hanks. This procedure allowed the removal of radioactivity not taken up by the tissue. After washing, 1 mL of water was given to the tissue to lysate the cells membranes. Following repetitive freeze–thaw cycles, cellular radioactivity was determined using a scintillation counter. We determined protein content using the method of Lowry et al. (1951).

[^{3}H] D-Aspartate release

Rat retinas (PN3, PN7, PN14) were incubated for 1 h in 1 mL of Hanks containing 1 μCi [^{3}H]-D-Aspartate and 10 μM D-Aspartate as a carrier as described in (Stutz et al., 2011). The medium was removed and the tissue washed four times with 1 mL Hanks. The tissue was then superfused with 0.5 mL of Hanks at 37 °C with additional drugs, as indicated. The superfusion was conducted by performing successive changes of 0.5 mL of the bathing medium at intervals of 10 min. Cellular radioactivity was assayed using a scintillation counter and results were normalized to percentage of neurotransmitter released according to (Kubrusly et al., 1998).

Western Blot assays

Rat Retinas (PN3, PN7 or PN14) exposed or not to 200 μM caffeine for 60 min were homogenized in a sample buffer (20 mM Tris base, 10 mM $MgCl_2$, 600 μM $CaCl_2$, 500 μM EGTA, 1 mM DDT). Then, protease inhibitor cocktail was added (1 mM PMSF, 5 μg/mL aprotinin, 2 μg/mL leupeptin) and 0.05% Triton X-100. Protein measurement was made through Bradford method. Samples were diluted in buffer containing 10% glycerol (v/v), 1% β-mercaptoethanol, 3% SDS and 62.5 mM Tris base, and heated for 5 min at 95 °C. We used 40 μg of protein from each sample in 10% SDS–PAGE and transferred to nitrocellulose membranes (0.2 μm pore). We then