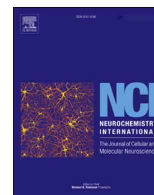
Neurochemistry International 108 (2017) 52–59

Contents lists available at ScienceDirect

Neurochemistry International

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



Characterization of the cystine/glutamate antiporter in cultured Bergmann glia cells

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A R T I C L E I N F O

Article history:
Received 14 September 2016
Received in revised form
27 January 2017
Accepted 20 February 2017
Available online 24 February 2017

Keywords:
Cystine/glutamate antiporter
Glutamate plasma membrane transporters
Signal transduction
Nitric oxide donors

A B S T R A C T

Glutamate, the major excitatory transmitter in the vertebrate brain is a potent neurotoxin through the over-stimulation of its specific membrane receptors. In accordance, a tight regulation of its extracellular levels by plasma membrane transporters is present. A family of excitatory amino acid transporters is expressed in neurons and glia cells and is responsible of the removal of the neurotransmitter from the synaptic cleft. Glial transporters account for more than 80% of the brain uptake activity. The cystine/glutamate antiporter is another plasma membrane-bound protein critically involved in glutamatergic transmission. Upon oxidative stress, it begins to pump out glutamate in exchange for cystine, mostly needed for glutathione production. Taking into consideration that all of these glutamate transporter proteins are present in glia cells that surround glutamatergic synapses, we reasoned that a functional coupling of them should exist to prevent an excitotoxic insult to the neighboring neuronal cells. To this end, we used the established model of chick cerebellar Bergmann glia cultures. Once we could establish the expression of the cystine/glutamate antiporter in our system, we characterized its kinetic properties and started to gain insight into its regulation and plausible coupling to other transporters. Exposure to glutamate reduces the uptake activity and favors a physical interaction with the excitatory amino acid transporter 1 and the Na^+-dependent neutral amino acids transporter 3. In contrast, treatment of the cultured cells with a nitric oxide donor such as sodium nitroprussiate augments the exchanger activity. Longer sodium nitroprussiate exposure periods down-regulates the cystine/glutamate protein levels. These results suggest that a coordinated interplay between glutamate transporters and exchangers takes place in glia cells to prevent excitotoxic insults.

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1. Introduction

L-Glutamate (Glu) is the major excitatory transmitter in the vertebrate brain, it exerts its actions through the activation of specific membrane receptors expressed both in neurons and glial cells (Gallo and Ghiani, 2000). Over-stimulation of neuronal Glu receptors results in an excitotoxic insult, prevented by the tight regulation of the extracellular levels of this amino acid. A family of excitatory amino acid transporters (EAAT) is responsible for the fast and efficient removal of the transmitter from the synaptic cleft. Even though these proteins are expressed in glia cells and neurons, the glial transporters account for more than 80% of the Glu uptake activity in the central nervous system (Danbolt, 2001).

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http://dx.doi.org/10.1016/j.neuint.2017.02.011
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Astrocytes are involved in a variety of brain functions such as extracellular ion balance, neurotransmitter metabolism and recycling, neurotrophic factors synthesis and release, among some other biochemical transactions. In glutamatergic synapses, astrocytes are compulsory participants, given the fact that these cells remove synaptically released Glu, which is mostly metabolized to glutamine (Gln), to be released back to the synaptic space *via* the inverse function of the sodium-dependent neutral amino acid transporter 3 (SNAT3) (Martínez-Lozada et al., 2013). Gln is taken up by the neuronal SNAT2 to be converted to Glu by neuronal glutaminase and packed into vesicles by the vesicular Glu transporters (VGLUT), completing the so-called *Glu/Gln shuttle* (Shank and Campbell, 1984). This shuttle is supported by a functional and physical interaction of EAATs and SNATs in Bergmann glia cells (Martínez-Lozada et al., 2013).

The Na^+-dependent Glu/aspartate transporter (GLAST/EAAT1) is the most abundant glial Glu transporter in the cerebellar cortex and it is highly expressed in Bergmann glia. These cells completely surround the glutamatergic synapses established between the axons of the granular cells (parallel fibers) and the dendrites of the Purkinje cells (Somogyi et al., 1990). A close and intimate interplay between Bergmann glia and surrounding neurons has been described and is related to Glu turnover, metabolic interdependence and differential gene expression (Martínez-Lozada and Ortega, 2015).

Although it has been traditionally assumed that Glu ambient concentrations are dependent upon EAATs function, in recent years it has become clear that glial uptake as well as its release are relevant for a proper glutamatergic transmission. In particular, a plausible involvement of the cystine/Glu antiporter x_c^- in Glu homeostasis has been recently described (De Bundel et al., 2011). This protein uptakes cystine and releases Glu in a 1:1 ratio. It is formed by two subunits: a 55 kDa polypeptide known as xCT (functional subunit) and 4F2hc linked by a disulfide bridge and is critically involved in the synthesis of the anti-oxidant glutathione (GSH) (Albrecht et al., 2010). In fact, exposure to a plethora of xenobiotics is linked to reactive oxygen species (ROS) production triggering x_c^- activity (Dal-Cim et al., 2016). Moreover, Glu itself has been shown to favor ROS synthesis and therefore x_c^- activity (Quincozes-santos et al., 2014), this cellular mechanism has to be tightly controlled, since Glu released might exacerbate its neurotoxic properties. With this in mind, we decided to investigate a plausible functional coupling of GLAST/EAAT1 with the cystine/Glu antiporter, specifically with the xCT subunit. To this end, we decided to use the established model of chick cerebellar Bergmann glia cultures (Ortega et al., 1991). After characterizing the expression and kinetic parameters of the exchanger, we established its regulation by nitric oxide and its co-immunoprecipitation with GLAST/EAAT1 and SNAT3, favoring the notion of the critical role of glia cells in Glu transmission.

2. Methods

2.1. Materials

Tissue culture reagents were obtained from GE Healthcare (Carlsbad, CA, USA). Quisqualate, D-aspartate, DL-*threo*-β-Benzyloxyaspartic acid (TBOA) and Glu were obtained from Tocris Bioscience (Minneapolis, MN, USA). Cyclic GMP, cyclic AMP, sodium nitroprusside (SNP), phorbol-12-myristate 13-acetate, Gln, Calbindin, DBA, and DPX were purchased from Sigma–Aldrich (St. Louis, MO, USA). $[^3H]$-L-Glutamate was acquired from Perkin Elmer (Boston, MA, USA). Horseradish peroxidase-linked anti-rabbit antibody and the enhanced chemiluminescence reagent (ECL) were obtained from Amersham Biosciences (Buckinghamshire, UK). xCT antibodies were acquired from Novus Biologicals (NB 300-318) (Littleton, CO, USA), and Abcam (ab175186) (Cambridge, MA, USA). The avidin-biotin complex (ABC) Peroxidase Standard Staining kit and agarose-coupled Protein A were purchased from ThermoFisher (Waltham MA, USA).

2.2. Cell culture and stimulation protocol

Primary cultures of cerebellar Bergmann glia cells (BGC) were prepared from 14-day- old chick embryos as previously described (Ortega et al., 1991). Cells were seeded in 6 or 24-well plastic culture dishes in Opti-MEM containing 2.5% fetal bovine serum, 2 mM Gln, and gentamicin (50 mg/ml) at 37 °C under standard conditions (5% CO_2 and 95% humidity) and used on the 4th–7th day after culture. Inhibitors were included 30 min prior to agonist addition. Glu and its analogues were added to the medium for the indicated time periods.

2.3. $[^3H]$-L-Glutamate uptake

Confluent BGC monolayers seeded in 24-well plates were washed three times to remove all non-adhering cells with 0.5 ml aliquots of solution A containing 25 mM HEPES-Tris, 130 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$. 0.8 mM $MgCl_2$, 33.3 mM glucose, and 1 mM NaH_2PO_4 at pH 7.4. Na^+-free solutions were prepared replacing NaCl with choline chloride. Inhibitors and competitors were included at the indicated time periods and added simultaneously with the substrate, followed by the addition of solution A containing 0.4 mCi/ml $[^3H]$-L-Glu. The uptake was stopped by rapid aspiration of the radioactive medium and each well was rinsed with ice-cold solution A. The monolayers were solubilized with 0.1 M NaOH for 2 h at room temperature. The radioactivity associated to the solubilized suspension was determined in a Perkin Elmer scintillation counter. Three independent experiments in quadruplicates were carried out. Statistical analysis was performed comparing against non-stimulated cells using a non-parametric Kruskal–Wallis test and Dunn's post-hoc test ($^*P < 0.01$, $^{***}P < 0.001$) using the Prism GraphPad software. Statistical analysis was performed comparing against non-stimulated cells using a non-parametric Kruskal–Wallis test and Dunn's post-hoc test, using GraphPad Prism software.

2.4. SDS-PAGE and western blot

Cells from confluent monolayers were harvested with phosphate-buffer saline (PBS) (10 mM K_2HPO_4/KH_2PO_4, 150 mM NaCl, pH 7.4) containing phosphatase inhibitors (10 mM NaF, 1 mM Na_2MoO_4 and 1 mM Na_3VO_4). The cells were lysed with RIPA buffer (50 mM Tris–HCl, 1 mM EDTA, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mg/ml aprotinin, 1 mg/ml leupeptin, 1% NP-40, 0.25% sodium deoxycholate, 10 mM NaF, 1 mM Na_2MoO_4 and 1 mM Na_3VO_4 pH 7.4). Cell lysates were denaturized in Laemmli's sample buffer, and equal amount of protein (50 μg as determined by the Bradford method) were resolved through a 10% SDS-PAGE and then electroblotted to nitrocellulose membranes. Blots were stained with Ponceau S stain to confirm that protein content was equal in all lanes. Membranes were soaked in PBS to remove the Ponceau S and incubated in TBS containing 5% dried skimmed milk and 0.1% Tween 20 for 60 min to block the excess of non-specific protein binding sites. Membranes were then incubated overnight at 4 °C with primary antibodies as indicated in each figure, followed by the respective secondary antibodies. Immunoreactive polypeptides were detected by chemiluminescence in a MicroChemi DNR-Bioimaging System. Experiments were done in quadruplicate. Four densitometry analyses

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