Neurochemistry International xxx (2017) 1–8

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

Edna Suárez-Pozos [a], Zila Martínez-Lozada [a], Orquidia G. Méndez-Flores [a], Alain M. Guillem [a], Luisa C. Hernández-Kelly [a], Francisco Castelán [b], Tatiana N. Olivares-Bañuelos [c], Donaji Chi-Castañeda [a, d], Mustapha Najimi [e], Arturo Ortega, Ph.D. [a, *]

[a] Laboratorio de Neurotoxicología, Departamento de Toxicología, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, Ciudad de México, 07000, Mexico
[b] Unidad Foránea Tlaxcala, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónama de México, Tlaxcala, Mexico
[c] Instituto de Investigaciones Oceanológicas, Universidad Autónoma de Baja California, Ensenada, 22860, Mexico
[d] Soluciones para un México Verde S.A de C.V, Santa Fé, Ciudad de México, 012010, Mexico
[e] Laboratoire de recherché en pédiatrie, Institut de Recherche Expérimentale et Clinique, Cliniques Universitaires St Luc, Université catholique de Louvain, 1200, Bruxelles, Belgium

ARTICLE INFO

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

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

ABSTRACT

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.

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).

* Corresponding author. Laboratorio de Neurotoxicología, Departamento de Toxicología, Cinvestav-IPN, Apartado Postal 14-740, México, DF 07000, Mexico.
E-mail address: arortega@cinvestav.mx (A. Ortega).

http://dx.doi.org/10.1016/j.neuint.2017.02.011
0197-0186/

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). [^{3}H]-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. [^{3}H]-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 [^{3}H]-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

Please cite this article in press as: Suárez-Pozos, E., et al., Characterization of the cystine/glutamate antiporter in cultured Bergmann glia cells, Neurochemistry International (2017), http://dx.doi.org/10.1016/j.neuint.2017.02.011

were performed comparing stimulated *versus* non-stimulated cells, using a non-parametric Kruskal–Wallis test and Dunn's post-hoc test using the GraphPad Prism Software.

2.5. Tissue preparation and immunohistochemistry

Cerebellum from 18-day-old chick embryos were collected and rinsed with 0.1 M PBS, followed by 4% paraformaldehyde diluted in PBS for 48 h and cryoprotected in sucrose solutions (10, 20 and 30% each time until the tissue sunk). Five micrometers thick sagittal sections were obtained from paraffin embedded tissue. The tissue slices were deparaffinized using standard procedures. Antigen retrieval was carried out in 100 mM citrate buffer pH 6 and heated in a microwave oven at 95 °C for 10 min. The sections were incubated in 3% H_2O_2 in PBS 0.1 M pH 7.4 for 30 min. To block unspecific binding, sections were incubated with normal goat serum in 0.3% of Triton- X-100 in PBS (PBST) for 1 h. Primary antibody incubation was performed for 72 h at 4 °C in blocking solution (anti-calbindin 1:5000, anti-xCT 1:250 or anti-KBP 1:250). Secondary antibodies (biotinylated anti-IgGs, 1:500 in PBST 0.1 M pH 7.4) were incubated for 2 h at room temperature. All sections were processed by the avidin-biotin complex method (ABC) (Thermo-Fisher) in a 1:200 dilution in PBS for 1 h at room temperature. The immunoreactivity was developed with 0.05% 3,3′-diaminobenzidibne (DAB) and 0.02% H_2O_2 in PBS 0.1 M pH 7.4. Finally, all sections were dehydrated in stepwise ethanol solutions (70, 96 and 100% respectively), coverslipped with DPX mounting medium (Sigma-Aldrich), and left to dry for 24 h at room temperature before further analysis.

2.6. Cell immunostaining

BGC were seeded on coverslips. Cells were fixed by exposure to ice-cold acetone for 10 min and air-dried for 1 h. Cells were rinsed with PBS twice and fixed for 10 min in 4% paraformaldehyde. Coverslips were rinsed again twice with TBS and one more time with TBS/Tween-20 (0.05%). Non-specific binding was prevented by incubation with 1% BSA in TBS (BSA/TBS) for 1 h. Cells were exposed to a 1:500 dilution of the primary antibody anti-xCT, in BSA/TBS over night at 4 °C, followed by the incubation with the respective fluorescein-labeled goat anti-rabbit antisera in BSA/TBS (1:500) for 2 h at room temperature. Preparations were mounted with Fluoroshield/DAPI. Cell preparations were examined under a fluorescence microscopy (Zeiss Axioscope 40 immunofluorescence microscope and the Axiovision software; Carl Zeiss, Inc., Thornwood, NY).

2.7. Immunoprecipitation

Cells from confluent monolayers were treated with 1 mM Glu for 30 min and harvested with PBS containing phosphatase inhibitors (10 mM NaF, 1 mM Na_2MoO_4 and 1 mM Na_3VO_4), and lysed with RIPA buffer. The total amount of protein was determined by the Bradford method. One mg of lysate was mixed with the respective agarose-coupled antibody (anti-xCT or anti GLAST) and incubated overnight at 4 °C. The immune-complexes were pelleted and rinsed 3 times with PBS 0.1 M. Proteins were denaturized in Laemmli's sample buffer. Negative controls and immunoprecipitated material were resolved through 10% SDS-PAGE and transferred to nitrocellulose membranes following the procedure previously described for SDS-PAGE and Western blot section.

3. Results

3.1. Expression of xCT in Bergmann glia

Previous reports have demonstrated the expression of xCT in neurons and glia (Gochenauer and Robinson, 2001). Based on these observations, we decided to investigate xCT expression in Bergman glia. To this end, tissue sections of 18-day chick embryos were obtained and incubated with anti-calbindin, anti-KBP and anti-xCT antibodies. The results are presented in panel A of Fig. 1. Anti-xCT immunoreactivity is present in the molecular layer with a localization that matches KBP staining, a specific marker of Bergmann glia cells (Ortega et al., 1991). It is important to note also xCT staining of Purkinje cells, reminiscent of the stainig with the specific marker calbindin (Pascual et al., 2014). As expected, our cultured cells also display anti-xCT immunoreactivity (Fig. 1, panel B).

In order to provide evidence of the x_c^- system functionality, [^{3}H]-L-Glutamate uptake was assayed, in presence (total uptake) or absence of Na^+ (system x_c^-). A Michaelis-Menten analysis revealed a *Vmax* value of 123.4 ± 1.7 pmol/mg*min for the total Glu uptake activity (EAATs + x_c^- system); whereas the *Vmax* values of 29.1 ± 2.6 were observed in Na^+-free media (x_c^- system) a *Vmax* value 88.4 ± 13.2 was calculated for EAATs (W-Wo). The K_M values obtained were 63.81 ± 7.3 μM, 32.50 ± 1.5 μM and 56.91 ± 1.1 μM respectively (total uptake, x_c^- and EAATs). These results demonstrate that around 20% of the total Glu uptake activity in BGC is carried out by the x_c^- system (Fig. 2, panel A). Previous reports using retinal ganglion cells 5 (RGC-5) demonstrated that quisqualic acid (Quis) acts as a competitive inhibitor of the x_c^- system since is transported by xCT (Chase et al., 2001; Dun et al., 2006). Accordingly, we decided to investigate the effect that Quis has on BGC [^{3}H]-L-Glutamate uptake in a Na^+-free media. Our data shows a reduction of approximately 40% in the *Vmax* (Fig. 2, panel B). As expected, in a Na^+-free medium, the Quis effect is dose-dependent with an IC_{50} of 3.99 ± 1.47 μM. It is pertinent to point that all values reported in these experiments represent the total uptake and have not been corrected for non-specific uptake or leakage. Taking together these results demonstrate the expression and functionality of the x_c^- system in cultured chick BGC.

3.2. X_c^- system substrate specificity and regulation in BGC

To evaluate the x_c^- system substrate specificity, we performed [^{3}H]-L-Glutamate uptake assays in absence of Na^+ but in presence of specific substrates, unlabeled amino acids Glu, Cystine ($(Cys)_2$), Cysteine (Cys), Alanine (Ala) and Leucine (Leu). When the uptake assay was performed in presence of substrates such as Glu, $(Cys)_2$ and Cys the [^{3}H]-L-Glutamate uptake was blocked by almost 50%, whereas no significant inhibitory effect was observed in presence of the rest of the unlabeled amino acids, demonstrating the identity of x_c^- as the transporter responsible of Glu uptake in a Na^+-free media (data not shown). At this stage, we decided to gain insight into some aspects of the regulation of the x_c^- system, to this end, we performed uptake experiments in the presence of several modulators. Given the fact that the Na^+-dependent Glu uptake in BGC, mediated by GLAST/EAAT1, is regulated by PKC, dbcAMP, dbcGMP and Glu itself (Flores-Méndez et al., 2015); we focused in these signaling pathways in [^{3}H]-Glu uptake experiments in Na^+-free media. An augmentation of the Glu uptake after exposure to 100 μM SNP, a donor of nitric oxide, is evident both in the long (24 h) or short time (4 h) of exposure (Fig. 3). Note that as expected hydrogen peroxide (H_2O_2) also potentiates [^{3}H]-L-Glutamate uptake after a long-term treatment.

Please cite this article in press as: Suárez-Pozos, E., et al., Characterization of the cystine/glutamate antiporter in cultured Bergmann glia cells, Neurochemistry International (2017), http://dx.doi.org/10.1016/j.neuint.2017.02.011

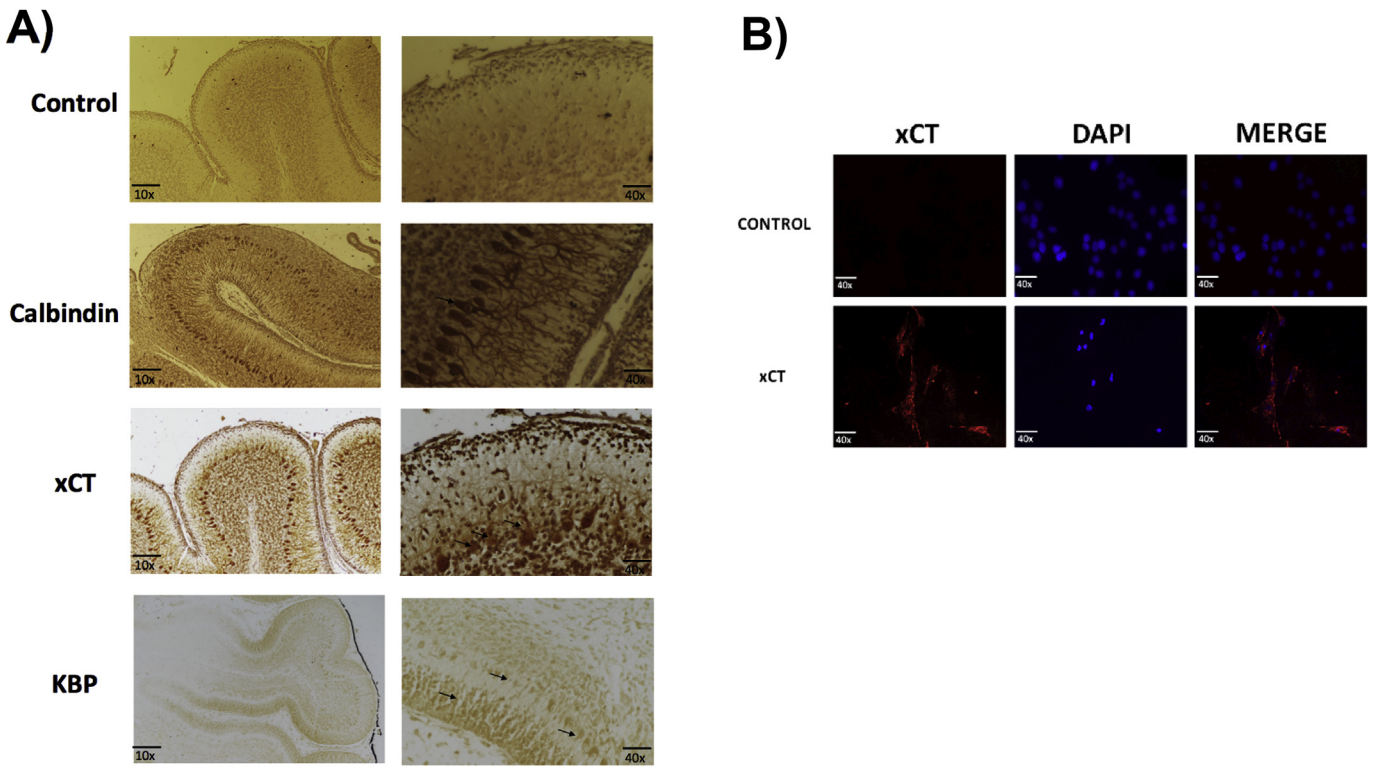

Fig. 1. xCT is expressed in Bergmann glia. Panel A: Fifty micrometers thick sagittal sections from embryonic E18 chick cerebellum were incubated with antibodies against the xCT protein in a dilution 1:250, the Purkinje cell marker calbindin (1:5000), and the Bergmann glia cells marker kainite binding protein (KBP) (1:250), the respective secondary antibodies were used in a 1:500 dilution. Dark precipitate from diaminobenzidine denotes detection of the proteins, absent in the control slide. Purkinje cell layer is identifiable by calbindin staining, while the xCT label was appreciated in the granular layer (astrocytes and Bergmann glia bodies), and along the Bergmann glia fibers in the molecular layer. Panel B: xCT immunoreactivity in Bergman glia cells (scale bar = 100 μm); primary antibody dilution used 1:500.

3.3. *xCT protein levels regulation in BGC*

The clear SNP regulation described above prompted us to investigate if the changes in uptake activity could be correlated to an increase in x_c^- protein levels. We were interested in gaining insight into the regulation level of the x_c^- system; as this system is composed by two proteins: a light chain xCT (the protein that performs the interchanger/channel task), and the heavy chain 4F2hc (a protein essential for xCT translocation to the cell membrane) (Lewerenz et al., 2013), we decided to evaluate if the observed changes in x_c^- activity were reflected at the protein levels of xCT. To achieve our aim, we first evaluated if a specific immunoreactivity using antibodies directed against xCT could be observed in our primary cultures of Bergmann glial cells, and in a positive control of mouse stomach (Fig. 4A). Although several immunoreactive bands were detected even after changing different methodologies for the blocking step, the predicted 55 kDa band is present as a faint doublet in the cultured cells, most possible reflecting a post-translational modification. Thereafter, cultured Bergmann glial cells were exposed to established Glu receptors and transporters modulators. Of relevance is the fact that a significant regulation of both the activity and the protein levels of xCT were modulated by the nitric oxide donor SNP, an agent known to up-regulate GLAST activity in Bergmann glia cells (Balderas et al., 2014). As shown in Fig. 4B, SNP exposure results in a biphasic effect, while a 4 h treatment increases (1.5 fold) the exchanger protein levels, after 24 h of exposure a reduction is evident. Please note the effect of dbcGMP, although apparently mimics the SNP effect after 4 h, at 24 h there is no decrease in x_c^- levels, most possibly reflecting the stability of dbcGMP. In any event, the dbcGMP effect is not statistically significant (Fig. 4B).

3.4. *Glu-dependent xCT protein complexes*

In the last few years, the characterization of the biochemical association of membrane proteins with complementary physiological roles has been described for Glu transporters (Martínez-Lozada et al., 2013; Robinson and Jackson, 2016; Rose et al., 2009). In this context, we decided to evaluate if Glu exposure would result in an association between GLAST and xCT. As depicted in Fig. 5, treatment with a 1 mM Glu concentration for 30 min is sufficient to increase the association of these two transporters. Note that we performed both GLAST and xCT immunoprecipitations. Given the reported association of GLAST and SNAT3 (Martínez-Lozada et al., 2013), we decided to test if in the immunoprecipitates, of control and Glu-exposed cells we could detect SNAT3, and as depicted in Fig. 5C and D, this was indeed the case, suggesting a functional and physical interaction between these transporter proteins.

4. Discussion

The fundamental role of astrocytes associated to glutamatergic synapses as neuronal partners in excitatory transmission is based mainly in their ability to clear the amino acid from the synaptic cleft by virtue of the reported abundance of Na^+-dependent transporters (Danbolt, 2001). In fact, it has been calculated that more than 80% of the total Glu uptake activity in the CNS is carried out by glial Glu transporters. Moreover, Glu turnover is likely to be dependent on the rather controversial *Glu/Gln shuttle* (Robinson

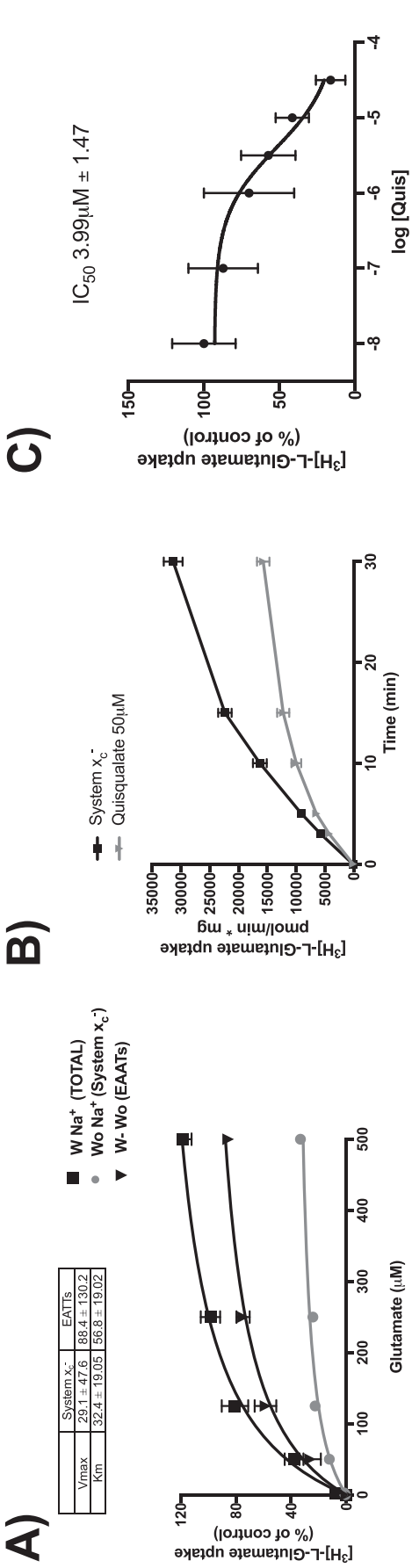

Fig. 2. [^{3}H] Glutamate transport is saturable and inhibited by Quisqualate in Bergmann glia cells. Panel A: Confluent BGC monolayers were exposed to different Glu concentrations for 30 min at room temperature plus 20 nM [^{3}H] Glutamate, in complete or Na^+ free assay buffer, the x_c^- component was calculated. Panel B: Quisqualate inhibition of [^{3}H] Glutamate uptake is concentration-dependent (Panel C). On Panels B and C, [^{3}H] Glutamate uptake was done in Na^+ free assay buffer. Three independent experiments in quadruplicate ± SEM are graphed for each data point. Statistical analysis was performed using a non-parametric Kruskal–Wallis test and Dunn's post-hoc test; $^*P < 0.01$, $^{***}P < 0.001$.

and Jackson, 2016). Apparently, the metabolic fate of Glu taken up by astrocytes depends on the model system used, i.e cortical or cerebellar astrocytes. Through the use of a primary culture of Bergmann glia cells from chick cerebellum, we have been able to demonstrate an activity-dependent interaction of GLAST/EAAT-1 and SNAT3 (Martínez-Lozada et al., 2013). Moreover, previous reports had shown that glutamate transporters function as signaling entities. On this matter, Martinez-Lozada and cols. in 2011 demonstrated that the exposure of Bergmann glia cultures to aspartate (the non-metabolized glutamate analog) increased mammalian target of rapamycin (mTOR) phosphorylation and activity, inducing Ca^{2+} influx and the activation of the phosphatidylinositol 3-kinase and protein kinase B. Furthermore, transporter signaling also augments the DNA binding activity of activator protein-1 (AP-1) and the up-regulation of the transcription of an AP-1 driven gene construct (Martínez-Lozada et al., 2011). In Bergman glia, two main subtypes of glutamate receptors have been defined: ionotropic (iGluRs) and metabotropic receptors (mGluRs). Ionotropic as well as metabotropic glutamate receptors are present in glia cells and have been postulated to participate in the regulation of cellular proliferation and differentiation, and in synaptic activity modulation (Bergles et al., 2010; Danbolt, 2001; Gallo and Ghiani, 2000; López-Bayghen and Ortega, 2011).

Another established role of astrocytes is the handling of reactive oxygen species (ROS), through anti-oxidant response, represented by the glial synthesis and release of GSH (Bakshi et al., 2015). The limiting substrate for the GSH synthesis is cysteine, which in glial cells is obtained by the hydrolysis of cystine, transported into the cells by the x_c^- system. Despite of the fact that we have previously evaluated Bergmann glia GSH synthesis in response to arsenite exposure (Castro-Coronel et al., 2011), a detailed characterization of system x_c^- had not been undertaken, therefore taking into consideration that Bergmann glia completely enwraps the most abundant excitatory synapse in the central nervous system (CNS), and that excessive Glu might trigger an anti-oxidant response in glia cells (Zepeda et al., 2008), we undertook the task of characterizing the expression of system x_c^- as well as some aspects of its regulation in our culture system.

Immunodetection of the xCT protein has been a difficult task due to the fact that none of the commercially available antibodies recognize a sole polypeptide band in Western blot analysis (see for example Fig. 4 A). Nevertheless staining of chick cerebellar slices with xCT decorated the cortex, in both in the granular and molecular layers, presumably in Bergmann glia fibers (Fig. 1). This assumption is based on the KBP signal, and correlates with previous reports of xCT in astrocytic cells (Bender et al., 2000; La Bella et al., 2007). It is pertinent to highlight the immunoreactivity in Purkinje cells and that Bergman glia bodies localize to the inner granular layer as described by Xu et al. (2013). Moreover, our findings properly are in line with those reported by Pow (2001), that using an antibody against aminoadipic acid (a specific substrate for the cystine-glutamate antiporter), the expression of the antiporter was shown mainly in the bodies and fibers of Bergmann glia cells throughout the molecular layer as well as an astrocytic labeling within the granular layer, reducing the controversy around the cross-reactivity that most of the commercially available xCT antibodies as reported previously (Van Liefferinge et al., 2016) (Fig. 1). Therefore, xCT identification in the cultured cells was not surprising, and accordingly, the predicted 55 kDa polypeptide could be identified *via* Western blot as shown in Fig. 4A. The presence of a doublet might reflect a post-translational modification such as glycosylation, although its characterization is beyond the scope of this communication. The controversy regarding specificity of xCT antibodies (Van Liefferinge et al., 2016) will certainly need a rigorous study of the biochemical properties of xCT in different cells

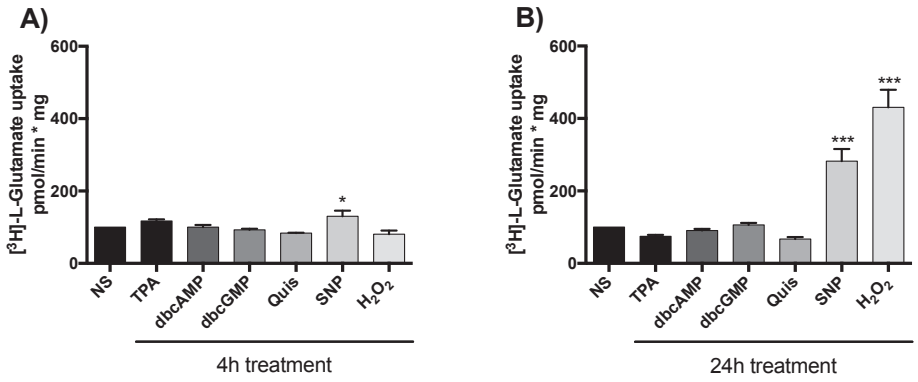

Fig. 3. The nitric oxide donor SNP increases [^{3}H] Glutamate uptake in Bergmann glia cells. Confluent BGC were stimulated for 30 min with 100 nm TPA, 500 μM dbcAMP or dbcGMP, 50 μM Quisqualate, 100 μM SNP or 0.0001% H_2O_2. For 4 (panel A) or 24 h (Panel B), and the [^{3}H] Glutamate uptake assay performed for 30 min. Results are the mean ± SEM of three independent experiments in quadruplicates. Statistical analysis was performed comparing against non-stimulated cells using a non-parametric Kruskal–Wallis test and Dunn's post-hoc test (*$P < 0.01$, ***$P < 0.001$).

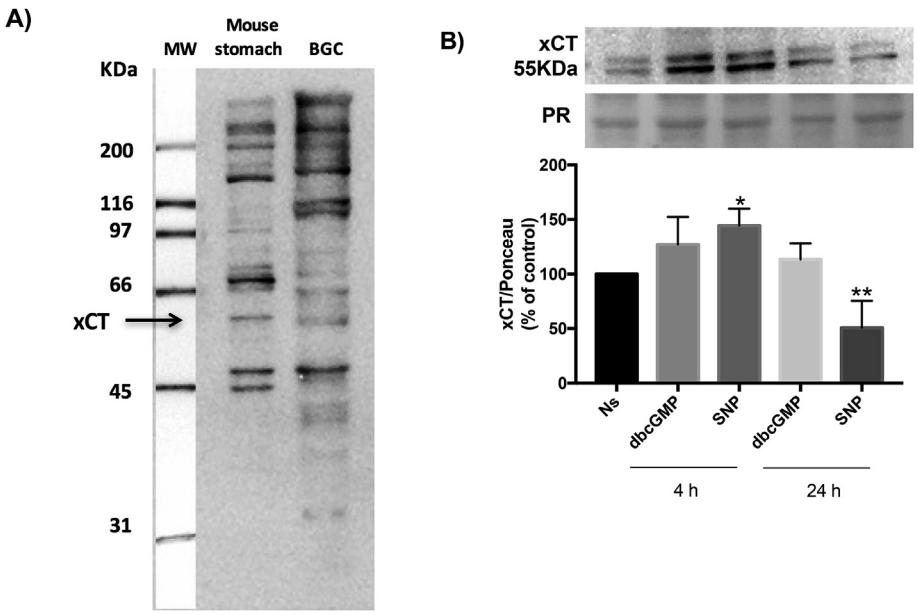

Fig. 4. SNP up-regulates xCT protein levels in cultured Bergmann glia. Panel A: Western blot analysis of mouse stomach and cultured Bergmann glia extracts with anti xCT antibodies. Equal amount of protein extracts (0.075 mg) were loaded in 10% SDS-PAGE gels and electroblotted as described under Materials and methods. A representative blot of four determinations is shown. Panel B: Monolayers of Bergmann glia cells were treated for 4 or 24 h with a fixed 100 μM SNP concentration, the cells were harvested and proteins extracts prepared Approximately 50 μg of protein per lane were analyzed *via* Western blot. The average of four densitometric analyses ± SEM is presented. A representative blot is presented on top of the graph. Statistical analysis was performed comparing against non-stimulated cells using a non-parametric Kruskal–Wallis test and Dunn's post-hoc test (*$P < 0.01$, ***$P < 0.001$).

and even in different species, moreover the production of new antibodies against different epitopes of the protein is a must.

The kinetic parameters established in the present communication are in line with the reported values (Bridges et al., 2012) reinforcing our conclusion of the expression and functionality system x_c^- in cultured Bergmann glia. In an effort to gain insight into system x_c^- regulation and its plausible role in Glu-transactions, we decided to evaluate the effect of the exposure of the cultured cells to established modulators of these Glu binding proteins. Of relevance is the fact that a significant regulation of both the activity and the protein levels of xCT was observed after the treatment with the NO donor SNP, an agent known to up-regulate GLAST activity in the cell system used in this study (Balderas et al., 2014). These results opened the possibility that a functional interaction of Glu transporters could be taking place. In this context, we decided to immunoprecipitate GLAST from control and Glu treated cells and analyze the presence of the light chain (xCT) in the immunocomplexes, a basal association between these proteins is present, but an increase in the association is present in treated cells (Fig. 5). At this stage, it was tempting for us to evaluate if SNAT3 could be also part of the transporters complex. The results suggest that after periods of intense synaptic activity, in which Glu accumulates, a complex of glial proteins is assembled for the proper handling and turnover of the excitatory transmitter. In this scenario, it is possible that overstimulation of the cerebellar parallel fibers would lead to a saturation of GLAST uptake activity which would favor the activation of AMPA and NMDA Bergmann glia receptors, resulting in ROS production, cystine uptake and Glu release *via* x_c^-, exacerbating Purkinje cell death as has been reported for the oral administration of diphenylarsinic acid in rodent cerebellum (Kato et al., 2007).

One would expect that the close association of x_c^- with GLAST could be linked to the immediate uptake of the Glu released *via* the

Please cite this article in press as: Suárez-Pozos, E., et al., Characterization of the cystine/glutamate antiporter in cultured Bergmann glia cells, Neurochemistry International (2017), http://dx.doi.org/10.1016/j.neuint.2017.02.011

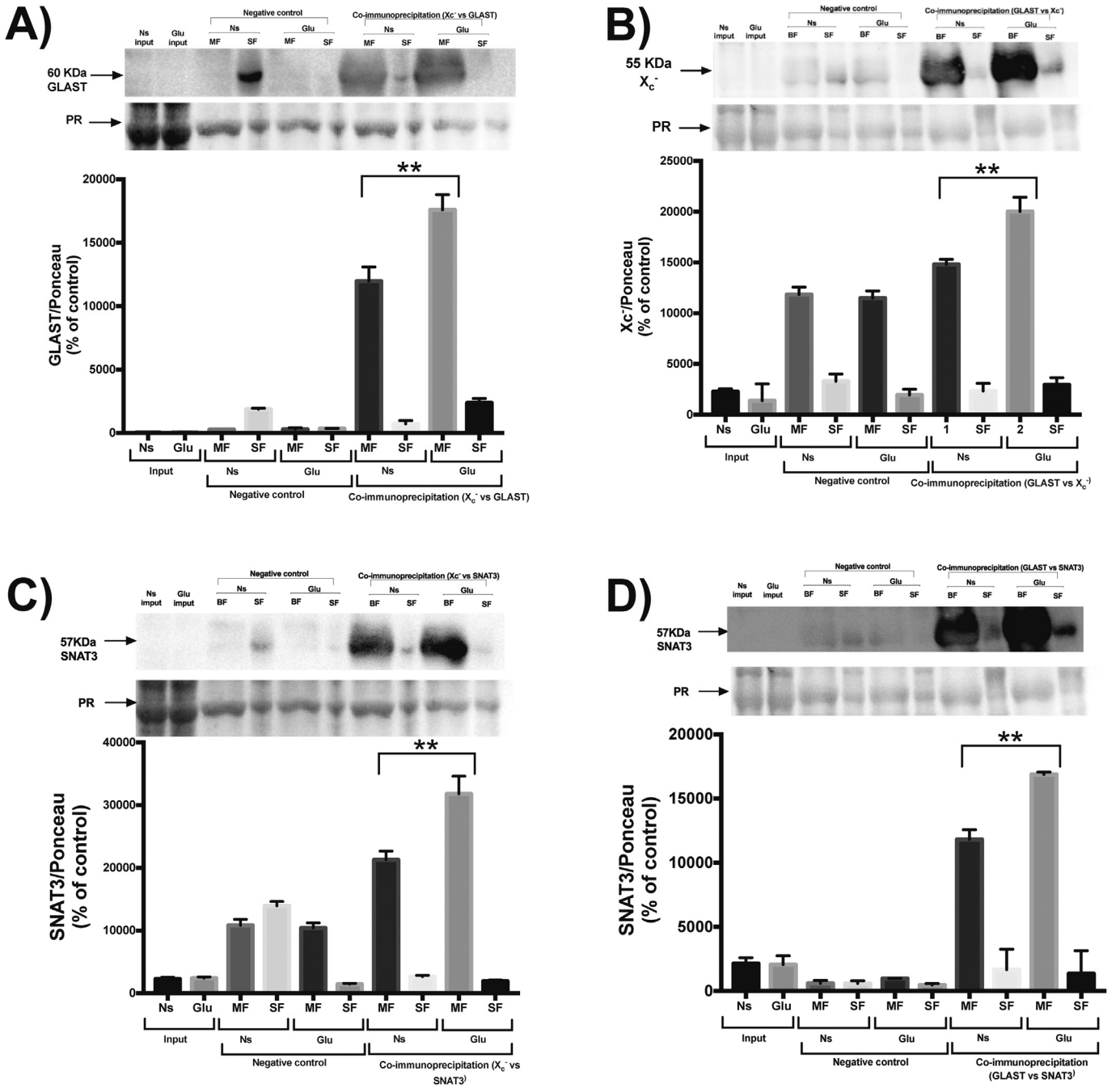

Fig. 5. Transporter complexes in control and Glu-treated cells. Panel A: Bergmann glia cells extracts from control or Glu-treated cells were immunoprecipitated with anti-xCT antibodies and the immunoprecipitate assayed by Western blot analysis with anti-GLAST antibodies. The opposite experiment is presented in Panel B. SNAT 3 is present in the xCT immunoprecipitate (Panel C) as well as in the GLAST immunoprecipitate (Panel D). Membrane fraction (MF), soluble fraction (SF), and Negative control correspond to all those samples prepared without coupling the antibody to the agarose beads. Representative gels of at least three independent experiments are shown. Graphs represent the statistical analysis performed comparing against non-stimulated cells using a non-parametric Kruskal–Wallis test and Dunn's post-hoc test ($^{*}P < 0.01$, $^{***}P < 0.001$) of at least three independent experiments.

exchanger, and its conversion and release as Gln. Experiments currently being carried out in our laboratories are aimed to the characterization of glial Glu binding proteins interactions.

In summary, in this communication we could demonstrate the expression of system x_c^- in Bergmann glia and provided preliminary evidence of a concerted interaction of Glu transporters.

Acknowledgments

This work was supported by grants from Conacyt-México (255087) to A.O and Conacyt-FNRS (210238) to M.N. and A. O. and by Soluciones para un México Verde to A.O. E.S-P is supported by a Conacyt-México postdoctoral fellowship. The technical assistance of Luis Cid and Blanca Ibarra is acknowledged.

Please cite this article in press as: Suárez-Pozos, E., et al., Characterization of the cystine/glutamate antiporter in cultured Bergmann glia cells, Neurochemistry International (2017), http://dx.doi.org/10.1016/j.neuint.2017.02.011

References

Albrecht, P., Lewerenz, J., Dittmer, S., Noack, R., Maher, P., Methner, A., 2010. Mechanisms of oxidative glutamate toxicity: the glutamate/cystine antiporter system xc- as a neuroprotective drug target. CNS Neurol. Disord. drug targets 9, 373–382.

Bakshi, R., Zhang, H., Logan, R., Joshi, I., Xu, Y., Chen, X., Schwarzschild, M.A., 2015. Neuroprotective effects of urate are mediated by augmenting astrocytic glutathione synthesis and release. Neurobiol. Dis. 82, 574–579. http://dx.doi.org/10.1016/j.nbd.2015.08.022.

Balderas, A., Guillem, A.M., Martínez-Lozada, Z., Hernández-Kelly, L.C., Aguilera, J., 2014. GLAST/EAAT1 regulation in cultured Bergmann glia cells: role of the NO/cGMP signaling pathway. Neurochem. Int. 73, 139–145. http://dx.doi.org/10.1016/j.neuint.2013.10.011.

Bender, A.S., Reichelt, W., Norenberg, M.D., 2000. Characterization of cystine uptake in cultured astrocytes. Neurochem. Int. 37, 269–276. http://dx.doi.org/10.1016/S0197-0186(00)00035-8.

Bergles, D.E., Jabs, R., Steinhäuser, C., 2010. Neuron-glia synapses in the brain. Brain Res. Rev. 63, 130–137. http://dx.doi.org/10.1016/j.brainresrev.2009.12.003.

Bridges, R.J., Natale, N.R., Patel, S.A., 2012. System xc- cystine/glutamate antiporter: an update on molecular pharmacology and roles within the CNS. Br. J. Pharmacol. 165, 20–34. http://dx.doi.org/10.1111/j.1476-5381.2011.01480.x.

Castro-Coronel, Y., Del Razo, L.M., Huerta, M., Hernandez-Lopez, A., Ortega, A., López-Bayghen, E., 2011. Arsenite exposure downregulates EAAT1/GLAST transporter expression in glial cells. Toxicol. Sci. 122, 539–550.

Chase, L.A., Roon, R.J., Wellman, L., Beitz, A.J., Koerner, J.F., 2001. L-Quisqualic acid transport into hippocampal neurons by a cystine-sensitive carrier is required for the induction of quisqualate sensitization. Neuroscience 106, 287–301.

Dal-Cim, T., Martins, W.C., Thomaz, D.T., Coelho, V., Poluceno, G.G., Lanznaster, D., Vandresen-Filho, S., Tasca, C.I., 2016. Neuroprotection promoted by guanosine depends on glutamine synthetase and glutamate transporters activity in hippocampal slices subjected to oxygen/glucose deprivation. Neurotox. Res. 29, 460–468. http://dx.doi.org/10.1007/s12640-015-9595-z.

Danbolt, N.C., 2001. Glutamate uptake. Prog. Neurobiol. 65 http://dx.doi.org/10.1016/S0301-0082(00)00067-8, 1–105.

De Bundel, D., Schallier, A., Loyens, E., Fernando, R., Miyashita, H., Van Liefferinge, J., Vermoesen, K., Bannai, S., Sato, H., Michotte, Y., Smolders, I., Massie, A., 2011. Loss of system xFormula does not induce oxidative stress but decreases extracellular glutamate in Hippocampus and influences spatial working memory and limbic seizure susceptibility. J. Neurosci. 31, 5792–5803. http://dx.doi.org/10.1523/JNEUROSCI.5465-10.2011.

Dun, Y., Mysona, B., Van Ells, T., Amarnath, L., Ola, M.S., Ganapathy, V., Smith, S.B., 2006. Expression of the cystine-glutamate exchanger (xc-) in retinal ganglion cells and regulation by nitric oxide and oxidative stress. Cell Tissue Res. 324, 189–202.

Flores-Méndez, M., Mendez-Flores, O.G., Ortega, A., 2015. Glia plasma membrane transporters: key players in glutamatergic neurotransmission. Neurochem. Int. 1, 1–10.

Gallo, V., Ghiani, C.A., 2000. Glutamate receptors in glia: new cells, new inputs and new functions. Trends Pharmacol. Sci. 7, 252–258.

Gochenauer, G.E., Robinson, M.B., 2001. Dibutyryl-cAMP (dbcAMP) up-regulates astrocytic chloride-dependent L-[3H]glutamate transport and expression of both system xc - subunits. J. Neurochem. 78, 276–286.

Kato, K., Mizoi, M., An, Y., Nakano, M., Wanibuchi, H., Endo, G., Endo, Y., Hoshino, M., Okada, S., Yamanaka, K., 2007. Oral administration of diphenylarsinic acid, a degradation product of chemical warfare agents, induces oxidative and nitrosative stress in cerebellar Purkinje cells. Life Sci. 81, 1518–1525.

La Bella, V., Valentino, F., Piccoli, T., Piccoli, F., 2007. Expression and developmental regulation of the cystine/glutamate exchanger (x c −) in the rat. Neurochem. Res. 32, 1081–1090. http://dx.doi.org/10.1007/s11064-006-9277-6.

Lewerenz, J., Hewett, S.J., Huang, Y., Lambros, M., Gout, P.W., Kalivas, P.W., Massie, A., Smolders, I., Methner, A., Pergande, M., Smith, S.B., Ganapathy, V., Maher, P., 2013. The cystine/glutamate antiporter system x $_c^-$ in health and disease: from molecular mechanisms to novel therapeutic opportunities. Antioxid. Redox Signal 18, 522–555. http://dx.doi.org/10.1089/ars.2011.4391.

López-Bayghen, E., Ortega, A., 2011. Glial glutamate transporters: new actors in brain signaling. IUBMB Life 63, 816–823. http://dx.doi.org/10.1002/iub.536.

Martínez-Lozada, Z., Guillem, A.M., Flores-Méndez, M., Hernández-Kelly, L.C., Vela, C., Meza, E., Zepeda, R.C., Caba, M., Rodríguez, A., Ortega, A., 2013. GLAST/EAAT1-induced Glutamine release via SNAT3 in Bergmann glial cells: evidence of a functional and physical coupling. J. Neurochem. 125, 545–554. http://dx.doi.org/10.1111/jnc.12211.

Martínez-Lozada, Z., Hernández-Kelly, L.C., Aguilera, J., López-Bayghen, E., Ortega, A., 2011. Signaling through EAAT-1/GLAST in cultured Bergmann glia cells. Neurochem. Int. 59, 871–879. http://dx.doi.org/10.1016/j.neuint.2011.07.015.

Martínez-Lozada, Z., Ortega, A., 2015. Glutamatergic transmission: a matter of three. Neural Plast. 2015, 1–11.

Ortega, A., Eshhar, N., Teichberg, V.I., 1991. Properties of kainate receptor/channels on cultured Bergmann glia. Neuroscience 41, 335–349.

Pascual, R., Valencia, M., Larrea, S., Bustamante, C., 2014. Single course of antenatal betamethasone produces delayed changes in morphology and calbindin-d28k expression in a rats cerebellar Purkinje cells. Acta Neurobiol. Exp. (Wars) 74, 415–423.

Pow, D.V., 2001. Visualising the activity of the cystine-glutamate antiporter in glial cells using antibodies to aminoadipic acid, a selectively transported substrate. Glia 34, 27–38. http://dx.doi.org/10.1002/glia.1037.

Quincozes-santos, A., Bobermin, L.D., Tramontina, A.C., Wartchow, K.M., Tagliari, B., Souza, D.O., Wyse, A.T.S., Gonçalves, C., 2014. Toxicology in Vitro Oxidative stress mediated by NMDA, AMPA/KA channels in acute hippocampal slices: neuroprotective effect of resveratrol. Toxicol. Vitr. 28, 544–551.

Robinson, M.B., Jackson, J.G., 2016. Astroglial glutamate transporters coordinate excitatory signaling and brain energetics. Neurochem. Int. 98, 56–71. http://dx.doi.org/10.1016/j.neuint.2016.03.014.

Rose, E.M., Koo, J.C.P., Antflick, J.E., Ahmed, S.M., Angers, S., Hampson, D.R., 2009. Glutamate transporter coupling to Na,K-ATPase. J. Neurosci. 29, 8143–8155.

Shank, R.P., Campbell, G.L., 1984. Amino acid uptake, content, and metabolism by neuronal and glial enriched cellular fractions from mouse cerebellum. J. Neurosci. 4, 58–69.

Somogyi, P., Eshhar, N., Teichberg, V.I., Roberts, J.D.B., 1990. Subcellular localization of a putative kainate receptor in Bergmann glial cells using a monoclonal antibody in the chick and fish cerebellar cortex. Neuroscience 35, 9–30. http://dx.doi.org/10.1016/0306-4522(90)90116-L.

Van Liefferinge, J., Bentea, E., Demuyser, T., Albertini, G., Follin-Arbelet, V., Holmseth, S., Merckx, E., Sato, H., Aerts, J.L., Smolders, I., Arckens, L., Danbolt, N.C., Massie, A., 2016. Comparative analysis of antibodies to xCT (Slc7a11): forewarned is forearmed. J. Comp. Neurol. 524, 1015–1032. http://dx.doi.org/10.1002/cne.23889.

Xu, H., Yang, Y., Tang, X., Zhao, M., Liang, F., Xu, P., Hou, B., Xing, Y., Bao, X., Fan, X., 2013. Bergmann glia function in granule cell migration during cerebellum development. Mol. Neurobiol. 1–12. http://dx.doi.org/10.1007/s12035-013-8405-y.

Zepeda, R.C., Barrera, I., Castelán, F., Soto-Cid, A., Hernández-Kelly, L.C., López-Bayghen, E., Ortega, A., 2008. Glutamate-dependent transcriptional regulation in Bergmann glia cells: involvement of p38 MAP kinase. Neurochem. Res. 33, 1277–1285. http://dx.doi.org/10.1007/s11064-007-9580-x.

Please cite this article in press as: Suárez-Pozos, E., et al., Characterization of the cystine/glutamate antiporter in cultured Bergmann glia cells, Neurochemistry International (2017), http://dx.doi.org/10.1016/j.neuint.2017.02.011