

Coupling of glutamate and glucose uptake in cultured Bergmann glial cells

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Abbreviations: (Glu) L-glutamate, (D-Asp) D- aspartic acid, (Gln) glutamine, (iGluRs) ionotropic glutamate receptors, (mGluRs) metabotropic glutamate receptors, (AMPA) α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, (NMDA) N- methyl-D-aspartate, (EAAT) excitatory amino acid transporter, (BGC) Bergmann glia cell, (GLAST) Glutamate Aspartate Transporter, (GLT1) Glutamate transporter 1, (ALS) Amyotrophic Lateral Sclerosis, (SNATs) sodium dependent neutral amino acid transporters, (GS) Gln synthetase, (GAP) phosphate-activated glutaminase, ($[^3\text{H}]2\text{DOG}$) $[^3\text{H}]2\text{-deoxy-D-glucose}$, (VGLUTs) vesicular Glu transporters, (GLUTs) Glucose transporters, (TTBS) Tris Buffered Saline, 0.1% Tween 20, (GLUT1) Glucose transporter 1, (GLUT3) Glucose transporter 3, (GLUTs) glucose transporters, (LDH) lactate dehydrogenase, (ANLS) Astrocyte Neuron Lactate Suttle.

HIGHLIGHTS

- Glucose uptake is enhanced by glutamate uptake in Bergmann glia
- An increase in plasma membrane glucose transporters is triggered by glutamate uptake
- Glucose transporters co-immunoprecipitate with glutamate transporters in an activity-dependent manner

ABSTRACT

Glutamate, the main excitatory neurotransmitter in the vertebrate brain, exerts its actions through specific membrane receptors present in neurons and glial cells. Over-stimulation of glutamate receptors results in neuronal death, phenomena known as excitotoxicity. A family of sodium-dependent, glutamate uptake transporters mainly expressed in glial cells, removes the amino acid from the synaptic cleft preventing neuronal death.

The sustained sodium influx associated to glutamate removal in glial cells, activates the sodium/potassium ATPase restoring the ionic balance, additionally, glutamate entrance activates glutamine synthetase, both events are energy demanding, therefore glia cells increase their ATP expenditure favouring glucose uptake, and triggering several signal transduction pathways linked to proper neuronal glutamate availability, *via* the glutamate/glutamine shuttle. To further characterize these complex transporters interactions, we used the well-established model system of cultured chick cerebellum Bergmann glia cells.

A time and dose-dependent increase in the activity, plasma membrane localization and protein levels of glucose transporters was detected upon D-aspartate exposure. Interestingly, this increase is the result of a protein kinase C-dependent signaling cascade. Furthermore, a glutamate-dependent glucose and glutamate transporters co-immunoprecipitation was detected. These results favour the notion that glial cells are involved in glutamatergic neuronal physiology.

Keywords: Glucose transporters, excitatory amino acid transporters, Bergmann glia, 2-deoxy D glucose.

Introduction

Glutamate (Glu) is the main excitatory amino acid neurotransmitter in the Central Nervous System (CNS) (Fonnum, 1984). A high percentage (80-90%) of brain synapses use it as their neurotransmitter (Braitenberg et al., 1998), therefore Glu is involved in almost every function of the brain, from sensory motor information and coordination to emotions and cognition. Glu exerts its actions through the activation of specific membrane receptors and transporters that are expressed both in neurons and glia cells. Glu receptors are divided into two main subtypes: ionotropic (iGluRs) and metabotropic receptors (mGluRs) (Hollmann and Heinemann, 1994).

Removal of Glu from the synaptic cleft relies in a family of sodium-dependent Glu transporters, known as excitatory amino acid transporters (EAATs) (Danbolt, 2001). Five different EAAT subtypes have been described, EAAT1/GLAST and EAAT2/GLT1 are preferentially expressed in glial cells and carry more than 80% of the total brain Glu uptake activity (Swanson, 2005). Within the cerebellar cortex, most of the Glu uptake takes place in Bergmann glial cells (BGC), these cells express exclusively EAAT1/GLAST, also known as Na⁺- glutamate/aspartate transporter (GLAST). In contrast, in most of all other CNS structures, EAAT2 or Glutamate transporter 1 (GLT-1) is the major Glu carrier, in fact, it is known that this transporter represents roughly 2% of total brain protein (Danbolt et al., 1998). It should be noted that this transporter is also present in presynaptic neurons in certain brain areas such as the hippocampus (Danbolt, 2001).

Glu extracellular levels are tightly controlled in order to prevent its toxicity to neurons and oligodendrocytes. Neurodegenerative diseases such as Amyotrophic Lateral Sclerosis (ALS) and Alzheimer's disease are related to an un-regulated excess of extracellular Glu (Sheldon and Robinson, 2007; Paul and de Belleruche, 2014).

It has long been proposed that glutamatergic transmission depends on a metabolic and energetic coupling between neurons and glia cells (Fatemi, 2008; Rojas, 2014; Volk et al., 2015). The neuronal availability of releasable Glu involves the proper function of glial proteins, Glu transporters, the enzyme glutamine (Gln) synthetase (GS) and the sodium dependent neutral amino acid transporters (SNATs). An accepted model of this coupling is the *so-called* Glu/Gln shuttle (Shank and Campbell, 1984). After an action potential, Glu released from the presynaptic terminal activates post-synaptic receptors and diffuses to the neighbouring glia cells, despite of the fact of presence of neuronal Glu transporters of the EAAT3 subtype, mainly due to their low expression, high affinity/low capacity characteristics (Danbolt, 2001). The abundance and capacity of glia Glu transporters is sufficient to clear extracellular Glu micromolar concentrations. Once internalized, Glu is mainly metabolized to Gln by means of the glia-enriched GS (Farinelli and Nicklas, 1992), accumulated Gln is released to the extracellular media through the inverse function of Gln transporters of the N family (SNAT 3,5). Presynaptic neurons take up Gln *via* the A subtype of Gln transporters (SNAT 1, 2) and through the action of phosphate-activated glutaminase (GAP) Gln is converted back to Glu that is packed in the presynaptic vesicles through the vesicular Glu transporters (VGLUTS) completing the cycle. Needless to say, inhibition of any of these proteins impairs glutamatergic synaptic transmission (Broer and Brookes, 2001).

It has been demonstrated that glia Glu uptake is linked to Gln release (Billups et al., 2013; Martinez-Lozada et al., 2013). Furthermore, an aspartate-dependent EAAT1-SNAT3 physical and functional interaction has been reported in BGC (Martinez-Lozada et al., 2013). Central to this coupling is the Na⁺/K⁺ ATPase, which also has been shown to associate with Glu transporters. The ATPase activity is needed to extrude the excess of Na⁺ ions that enter the cell

together with Glu *via* EAAT1/GLAST (Rose et al., 2009). The energetic coupling between astrocytes and neurons was suggested from experiments using cultured cortical glia exposed to Glu for short time periods. It became evident that astrocytes release lactate after an increased sodium-dependent Glu uptake (Pellerin and Magistretti, 1994). An Astrocyte-Neuron Lactate Shuttle (ANLS), was proposed and described in a number of systems (Jakoby et al., 2014).

We hypothesize that within the cerebellar cortex, BGC could be energetically coupled to the glutamatergic synapses surrounded by them. In such a scenario, glucose uptake would be favoured. To gain insight into this possibility, we decided to characterize [^3H]2-deoxy-D-glucose ([^3H]2DOG) uptake in primary cultures of chick cerebellar BGC. These cells completely enwrap the most abundant glutamatergic synapse in the encephalon, the one formed by the parallel fibers and the Purkinje cells in the cerebellar cortex (Somogyi et al., 1990).

The glucose transporter protein family (GLUTs) is integrated by fourteen members, from which class I isoforms have been better described in brain (Augustin, 2010). Tissue and cell specificity have been established for GLUTs. The major glucose transporter expressed in granular cells and neuronal cell lines is GLUT3, mainly in the plasma membrane. Interestingly, low levels of GLUT3 have been identified in rat cortical astrocytes (Maher et al., 1991). In general terms, it is quite accepted that GLUT1 is mainly found in glial cells and GLUT3 in the neuronal counterpart (Brekke et al., 2015).

Upon treatment of BGC with its specific EAAT ligand, D-aspartate, a time and dose-dependent increase in [^3H]2DOG uptake is observed. An increase of available glucose transporters in the plasma membrane is likely to be the responsible for the enhanced uptake. Moreover, an interaction between Glu and glucose transporters could be detected. These results support the involvement of glia/neuronal interactions in glutamatergic transmission.

2. Methods

2.1 Materials

Tissue culture reagents were obtained from GE Healthcare (Carlsbad, CA, USA). Deoxy-D-glucose, 2-[1,2-³H (N)] (specific activity 20 Ci/mmol) was from Perkin Elmer (Boston, MA, USA). L-Gln, D-Asp, Glu and 2-Deoxy-D-glucose (2DOG) were obtained from Sigma-Aldrich, Mexico. Bisindolylmaleimide I (Bis I) from Calbiochem, (EMD Millipore, Darmstadt, Germany), H-9 dihydrochloride, KB-R7943 mesylate, DL-threo-b-Benzoyloxyaspartic acid (TBOA), threo-b-hydroxyaspartate (THA) were from Tocris-Cookson (St. Louis, MO, USA).

Goat polyclonal anti-GLUT1 (sc-1605) and anti-GLUT3 (sc-7581) were from Santa Cruz Biotechnology, Dallas, TX USA. Monoclonal anti-calbindin D (C-8666) was from Sigma-Aldrich, St. Louis, MO, USA), anti-kainate binding protein (KBP) and anti-GLAST anti-serum were produced and characterized in our laboratory (Martinez-Lozada et al., 2013). Monoclonal anti-actin antibody was kindly donated by Prof. Manuel Hernández (Cinvestav-IPN). Protein A and G coupled to agarose particles, horseradish peroxidase-coupled anti-mouse, anti-goat, anti-rabbit antibodies, and the Enhanced Chemiluminescence reagent, were obtained from GE Healthcare Life Sciences (Mexico DF). EZ-Link® Sulfo-NHS-LC-Biotin and agarose-coupled Streptavidin were from Thermo Scientific. All other chemicals were purchased from Sigma (St. Louis, MO, USA).

2.2 Cell culture and stimulation protocol

Primary cultures of cerebellar BGC were prepared from 14-day-old chick embryos, with subtle adjustments from our previously described protocol (Ortega et al., 1991). Briefly, trypsin and mechanically dissociated cells were sedimented consecutively two times, then plated in 24-well and 6-well plastic culture dishes in OPTIMEM containing 4% foetal bovine serum (FBS),

gentamicin (50 μ g/ml) and used on the 3rd to 5th day after plating. Before any treatment, assay buffer solution containing 25 mM HEPES-Tris, 130 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 0.8 mM MgCl₂, 5 mM glucose and 1 mM NaH₂PO₄ at pH 7.4 was replaced on the confluent monolayers. Experiments with different ionic conditions in the assay buffer solution included Tris-HCl for NaCl replacement and 0.1 mM EDTA addition for without calcium condition. Inhibitors and blockers were incubated 20 min before stimulation.

2.3 [³H]-2-D-deoxyglucose uptake

Confluent BGC monolayers seeded on 24-well plates were stimulated in assay buffer solution, following to 30 min glucose analogue uptake in the same solution containing 0.4 μ Ci/ml of [³H]2DOG (20 nM) plus 1- 5 mM of non-labelled 2DOG. When indicated, NaCl was replaced by Tris-HCl or calcium removed and EDTA added to assay buffer solution. The [³H]2DOG influx was initiated by the addition of 0.25 ml uptake solution per well. Kinetic parameters were estimated by uptake assay with increasing concentrations of unlabelled 2DOG: 0, 0.1, 0.5, 1 and 2 mM plus a fixed concentration of labelled 2DOG (20 nM), the uptake time was at 30 min. The reaction was stopped by aspirating the radioactive medium and washing thoroughly with ice-cold assay buffer solution. The BGC monolayers were lysed with 0.1 M NaOH at room temperature, an aliquot of sample was used for protein determination by the Bradford method, and radioactivity from samples was determined by liquid scintillation counting in a Perkin Elmer scintillation counter. Experiments were carried at least three times with quadruplicate determinations.

2.4 SDS-PAGE and Western blots

Cells from confluent monolayers were treated and harvested with phosphate buffer saline (PBS) (10.1 mM Na_2HPO_4 , 1.8 mM KH_2PO_4 , 2.7 mM KCl, 137 mM NaCl, pH 7.4) containing phosphatase and general protease inhibitors (10 mM NaF, 1mM Na_2MoO_4 and 1 mM Na_3VO_4 and 1 mM phenylmethylsulfonyl fluoride –PMSF-). The cells were pelleted at top speed in a refrigerated microfuge, lysed with 50 mM Tris-acetate buffer, pH 7 plus 5 mM EDTA, 1 mM PMSF, 1 mM Na_3VO_4 , 1 $\mu\text{g/ml}$ aprotinin, 1 $\mu\text{g/ml}$ leupeptin and 0.2% sodium dodecyl sulphate (SDS). Protein concentration was determined by the Bradford method, using bovine γ -globulins for standard curve. Cell lysates were denatured by boiling for 5 min in Laemmli's sample buffer, resolved through 10% SDS-PAGE and then electro-blotted to nitrocellulose membranes. Blots were stained with Ponceau S solution to confirm equal protein content in all lanes. Membranes were soaked in PBS to remove the Ponceau S and incubated for 30 min in 5% non-fat dried milk solution in TTBS (Tris Buffered Saline, 0.1% Tween 20) to block the excess of non-specific protein binding sites. Then membranes were incubated overnight with the primary antibodies at 4°C. Goat anti-GLUT1 (1:500), goat anti-GLUT3 (1:500) and rabbit anti-GLAST (1:2000), followed by secondary antibodies incubation for 2 hours at room temperature. Immunoreactivity was detected by chemiluminescence with a MicroChemi (DNR Bio-Imaging System) charge-coupled device (CCD) imager. Densitometric analysis were performed with Image J64 application (Schneider et al., 2012) and data analysed with the Prism 5, GraphPad Software (San Diego, CA, USA).

2.5 Plasma membrane protein biotinylation

To establish the GLUT1 plasma membrane levels, two 35-mm culture wells (from a six-well culture plate) were used for each experimental condition (n=3). After treatment, the wells were washed with 1 ml of ice-cold PBS and the BGC monolayers incubated with EZ-Link Sulfo-NHS-LC-Biotin (0.14 mg/ ml or 0.25mM) solution in PBS calcium-magnesium (1mM Ca^{2+} and 0.5 mM Mg^{2+}) for 30 min at 4°C under gentle agitation. Non-bound biotin was removed by washing the culture plates three times with ice-cold 0.1 M L-Glycine in PBS calcium-magnesium. Subsequently, the cells were scraped and lysed in approximately five times the pellet volume with lysis buffer (50 mM Tris-acetate buffer, 5 mM EDTA, pH 7, 0.2% (v/v) SDS plus protease and phosphate inhibitors). The lysate was centrifuged at high speed in a table top centrifuge for 5 min at 4°C, and the protein concentration from the supernatant were measured by the Bradford method. At least 300 µg of protein were mixed with 30 µl of streptavidin-coupled agarose particles. The suspension was incubated at 4°C overnight with slight agitation. The first supernatant was separated and used as the non-biotinylated intracellular protein fraction; meanwhile the agarose-streptavidin particles were washed three times with 0.5 ml of 0.1% (v/v) Triton X-100 PBS, centrifuged at 16000 x g for 10 min at 4°C. Finally inputs, supernatant and pellets were denatured by boiling for 5 min in Laemmli's sample buffer. Equal amount of proteins was resolved through SDS-PAGE.

2.6 Immunoprecipitation

Cell lysates were immunoglobulin-cleared with 3 µl of protein A or G coupled to agarose, under agitation for 30 min at 4 °C. Then protein content was determined by the Bradford method. Agarose-coupled antibodies were incubated (overnight, 4 °C) with 300 µg of pre-adsorbed protein samples. The immune-complexes were pelleted, washed three times with PBS and boiled

for five minutes in Laemmli's sample buffer. Control and immunoprecipitated material was resolved through 10% SDS-PAGE and transferred to nitrocellulose membranes.

2.7 Immunofluorescence and immunohistochemistry

BGC primary cultures were seeded on glass coverslips following the procedure described above. Cells were rinsed twice with PBS, fixed with 4% paraformaldehyde for 10 min at room temperature, residual aldehydes were quenched with 0.05 M NH_4Cl solution for 5 min. Cell membranes were permeabilized with PBS containing 0.2% Tween 20 for 15 min, non-specific binding sites were blocked with 1% bovine serum albumin (BSA) and 0.3 M L-Gly in PBST (PBS containing 0.1 % Tween 20) solution for 30 minutes. Cells were incubated overnight at 4°C with primary antibodies: Goat anti-GLUT1 (1:250), goat anti-GLUT3 (1:250) and rabbit anti-KBP (1:500). After three washes, cells were incubated for 1 hour with corresponding fluorescent secondary antibodies dissolved in PBS containing 0.1% BSA: Alexa Fluor 488 donkey anti-goat IgG 1:1000 (A11055), TRITC goat anti-rabbit IgG 1:200 (81-6114) and TRITC goat anti-mouse IgG 1:200 (81-6514). After washing out the secondary antibodies, cell preparations were mounted in Fluoroshield with DAPI (F6057, Sigma-Aldrich) mounting medium. Preparations were analysed in a Leica TCS SP8 confocal platform.

For immunohistochemistry, post-natal day 18 chick cerebella was removed and placed immediately in cold PBS. The tissue was washed once in cold PBS to remove blood and placed in 4% paraformaldehyde in PBS (pH 7.4) for one hour. The fixative was changed once and the tissue was left at 4°C for 48 h. The cerebella were cryoprotected successively in 10%, 20%, and 30% sucrose in PBS and sagittally sectioned at 50 μm with a cryostat (Microm International GmbH, Walldorf, Germany).

For immunolabeling tissue was washed profusely in PBS to remove excess aldehydes and then incubated 10 min in 1.8% hydrogen peroxide solution to remove endogenous peroxidase activity. Non-specific antibody binding was blocked by incubating the sections in blocking solution (3% goat serum in PBS containing 0.3% Triton, PBT) for 1 h at 25°C. Sections were incubated at 4°C for 48 h with primary antibodies; mouse anti-calbindin (1:5000), anti-KBP (1:2500), anti-GLUT1 and anti-GLUT3 (1:500) in blocking solution. The sections were washed three times in PB, and placed in biotinylated anti-mouse secondary antibodies (1:1000; Vector Laboratories, Inc., Burlingame, CA, USA) for 3 h (for calbindin), anti-rabbit secondary antibodies for KBP, and anti-goat secondary antibodies for GLUT1 and GLUT3. A 1 h incubation with the avidin-biotinylated horseradish peroxidase complex (1:250; Vector Labs) followed. The antibody-peroxidase complexes were revealed with a solution containing 0.05% diaminobenzidine, nickel sulfate (10 mg/mL; Fisher Scientific, Pittsburg, PA, USA), cobalt chloride (10 mg/mL; Fisher Scientific), and 0.01% hydrogen peroxide, which produced a black–purple precipitate. Sections were mounted onto gelatin-subbed slides, dehydrated, and cleared in Hemo-De (Fisher Scientific) with Permount mounting medium. The sections were analysed in an Olympus BX41 microscope.

2.8 Statistical analysis

Data are expressed as the mean (average) \pm standard error (SEM). A one-way analysis of variance (ANOVA) was performed to determine significant differences between conditions. When this analysis indicated significance ($= / > 0.05$ level), a Dunnett's multiple comparison analysis was used to determine what conditions were significantly different from each other with the Prism 5, GraphPad Software (San Diego, CA, USA).

3. Results

3.1 BGC express GLUT1 and GLUT3

The possibility that glutamatergic neurotransmission is linked to glia metabolism, has been suggested since 1998 (Pellerin et al., 1998). Despite of these contributions, the molecular events associated to this coupling are far from being established. With this in mind we decided to take advantage of the well-established model of primary cultures of chick cerebellar BGC (Ortega et al., 1991). To this end, we first decided to evaluate the expression of the major CNS glucose transporters: GLUT1 and GLUT 3. As clearly shown in Figure 1 panel A, the characteristic 54 kDa protein band is present in both immunoblots, but as expected, an apparent enrichment of the glial transporter GLUT-1 is evident. Immunochemical evidence of the presence of both transporters in our cultured cells is presented in panel B of Figure 1. Note also the expression of the BGC marker, the kainate binding protein, KBP (Somogyi et al., 1990). Furthermore, immunohistochemical evidence of the expression of both glucose transporters at postnatal day 18 cerebellar slices is provided in panel C of Figure 1, again, GLUT-1 expression is more abundant than that of GLUT3 in BGC glia *in situ*.

3.2 Functional characterization of glucose uptake in BGC

The glucose uptake process was characterized in BGC monolayers. As depicted in Figure 2 panel A, a time dependent [^3H]2DOG uptake could be measured with a peak at 20 min and remains stable for 30 min. All subsequent experiments were performed for an uptake time of 30 min. When the concentration of [^3H]2DOG was varied, the kinetic parameters could be calculated, and a K_M of 1.3 mM and a V_{max} of 3112 pmol/mg prot/min was obtained, values that are in the

range of the reported values for GLUT 1 (Vannucci et al., 1997; Carruthers et al., 2009) (Figure 2, panel b).

Treatment of the cultured cells with 1 mM Glu or D-Asp results in a 30% increase in [^3H]2DOG uptake (Figure 3, panel A). Since these results could be consequence of a saturation of the Glu transporter, [^3H]2DOG uptake was performed in the presence of different D-Asp and Glu concentrations, and EC_{50} values of 11.7 and 9 μM respectively, were estimated (Figure 3, panels B and C). Taking into consideration that the EC_{50} aforementioned fall in the same range as EAAT1/GLAST, K_M of 24 μM for D-Asp uptake (Ruiz and Ortega, 1995), the most plausible explanation for the recorded increase in [^3H]2-DOG uptake is related to Glu uptake through EAAT1/GLAST that triggers an augmentation of GLUTs insertion in the plasma membrane.

3.3 GLAST-dependent increase in plasma membrane GLUT-1

In order to clarify if the increased [^3H]2-DOG uptake upon Glu exposure is related to an increase in glucose transporters in the plasma membrane, we decided to perform two different set of experiments: kinetic characterization in cells pre-treated with 1 mM Glu and biotinylation experiments of control and treated cells. Indeed, kinetic analysis of the Glu effect demonstrates a 2-fold increase in V_{max} favoring the notion of an increase in glucose transporters in the plasma membrane (Figure 4, panel A). A definite proof of our interpretation was established *via* biotinylation experiments. As clearly seen in panel B of Figure 4, Glu treatment results in a two-fold increase in GLUT-1 transporters in the plasma membrane. Besides the GLUT1 signal, EAAT1/GLAST was detected in the biotinylated fraction, alike α -actin detection in the supernatant fraction (Figure 4, panel C).

3.4 GLAST signalling of [^3H]2-deoxyglucose uptake augmentation

It has been previously reported that Glu transporters act as signal transducers (Martinez-Lozada et al., 2011; Maria Lopez-Colome et al., 2012). In order to delineate the biochemical events inherent to the described increase in plasma membrane GLUT1 levels, we first decided to remove the Na^+ ions from the assay buffer, in a effort to rule out the involvement of Glu receptors. As shown in panel A of Figure 5, the equimolar substitution of NaCl with Tris-HCl prevents completely the Glu effect ruling out any involvement of Glu receptors, as expected the D-Asp effect is also blocked. It could be argued that removal of Na^+ is not sufficient to prevent Glu receptors activation, but since D-Asp mimics the Glu effect, this possibility is remote. Taking into consideration that the insertion/removal of plasma membrane proteins might depend on cytoskeleton arrangements (Sullivan et al., 2007; Fehon et al., 2010; He et al., 2014), and that Ca^{2+} plays a major role in cytoskeletal organization, among other intracellular biochemical transactions, we decided to evaluate the participation of this ion in the described effect. The results are presented in panel B of Figure 5, the removal of Ca^{2+} prevents the EAAT1/GLAST dependent increase in [^3H]2DOG uptake.

As has already been described, EAAT1/GLAST mediated uptake triggers in the reversal function of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (Martinez-Lozada et al., 2011), therefore it was not surprising that KBR7943, a blocker of this anti-port, hampers the D-Asp effect. The involvement of the Ca^{2+} /diacylglycerol dependent protein kinase (PKC) was deduced from the ability of 3-(1-(3-(Dimethylamino)propyl)-1H-indol-3-yl)-4-(1H-indol-3-yl)-1H-pyrrole-2,5-dione (Bis I) to prevent the D-Asp effect (Figure 5, panel C). Finally, we decided to investigate if the GLUTs up-regulation is related to the uptake process itself, to this end we treated the cells with the transportable EAAT blocker THA, and as depicted in panel D of Figure 5, a modest increase in

[³H]2DOG is present, whereas with the non-transportable EAAT blocker TBOA, no increase in glucose uptake is obtained.

3.4 Biochemical interaction between GLAST and GLUT1

The results described thus far, clearly suggest a functional interplay between the Glu and glucose uptake processes. To gain insight about this plausible interaction, we performed co-immunoprecipitation experiments in control and treated cells. As represented in panels A and B of Figure 6, exposure of BGC to 1 mM Glu or D-Asp results in a significant association between EAAT1/GLAST and GLUT1, both revealed through the immunoprecipitation of either of the transporters and the detection of the other by Western blot analysis. Finally, in an effort to strengthen our understanding about the molecular mechanisms by which this GLAST-GLUT1 interaction takes place, we decided to measure total GLUT1 and GLUT3 total protein amount via Western blots in cells treated for 30 min with 1 mM Glu or 1 mM D-Asp. The results are presented in panels C and D of Figure 6: an increase in total GLUT1 and GLUT3 protein levels is detected, suggesting either an increase in glucose transporters translation or a decrease the glucose transporters degradation. Taken together our results suggest that synaptic released Glu modulates glia cell physiology triggering the plastic changes needed for proper Glu turnover and glycolytic metabolism.

Discussion

Since the decade of the 70s of the last century, in which the expression of neurotransmission related proteins was detected in glial cells (Henn and Henn, 1980), a putative role of glia cells in transmitter-mediated information transfer has been suggested. Nevertheless, much of what has been speculated and reviewed about the “*tripartite synapse*” refers to how glia cells alter synaptic function (Navarrete and Araque, 2014). Much less has been speculated about the consequences of neuronal signaling in glia physiology (Martinez-Lozada and Ortega, 2015). In this context, the use of suitable preparations is crucial to advance in our understanding of the molecular consequences of Glu and/or GABA exposure of glia cells. Glutamatergic as well as GABAergic transmission require the involvement of glia cells to efficiently clear the synaptic cleft and to recycle the transmitter through Glu/Gln shuttle (Danbolt, 2001; Schousboe and Waagepetersen, 2006; Eulenburg and Gomeza, 2010).

The present contribution is inserted in this problematic, what are the metabolic and signaling consequences of Na⁺-dependent Glu uptake in glia cells that could impact into the continuous their dialogue with neurons? To this end, we focused into glucose transport for two reasons, the first one being the fact that Glu exposure of BGC is linked to a transient decrease of protein synthesis (Barrera et al., 2008) indicative of an energetic failure upon increased Glu transport. The other reason is the reported glia enrichment of the type 5 isoform of lactate dehydrogenase (LDH) that favours lactate accumulation and its extrusion, giving support to the ANLS (Pellerin and Magistretti, 2012).

The first approach was to characterize the glucose transporters present in our model system. We were able to detect GLUT1 and GLUT3 in Bergman glia *in situ* as well as in cultured cells (Fig 1). It should be noted that GLUT3 is mainly present in neurons, although taking into

consideration that BGC has progenitor characteristics (Patten et al., 2006) it is not surprising that we detected this transporter. Moreover, it has been reported that in certain astrocytic populations GLU3 is present (Maher et al., 1991) and that it is inducible by nitric oxide (Cidad et al., 2001). Both GLUTs have an estimated molecular weight of approximately 54 kDa in chick tissues, however GLUT1 has two distinct molecular forms of an apparent molecular weight of 55 and 45 kDa, that differ only by their glycosylation pattern (Birnbaum et al., 1986), both forms are present in our cultured cells (Fig. 4 panel C).

To characterize the transport process, we measured [^3H]-2DOG transport. A time-dependent increase in the amount of radioactivity incorporated was found (Fig. 2A) in agreement with other cell systems (Cidad et al., 2001; Jakoby et al., 2014). To determine the kinetic constants of the transport, we used a 30 min uptake time. It can be argued that the kinetic parameters should be determined in the linear phase of the transport, although the low specific activity of [^3H]2DOG and the low affinity of the transport does not allow to perform such an experiment. In any case the parameters found are in line with previous publications (Colville et al., 1993) (Carruthers, 1990).

We could establish (Fig. 3A), as it has been reported for other astroglia cultures, that Glu increases glucose uptake (Pellerin and Magistretti, 1994), but the fact that we use a preparation of glia cells that we know that *in situ* surrounds glutamatergic synapses allows us to elaborate into a detailed molecular mechanism that overlap two of the most characterized astrocytic functions: neurotransmitter removal and glucose uptake (review in (Kirischuk et al., 2015)). Some arguments presume that Glu uptake may not induce an increase glucose uptake in astrocytes, since Glu total oxidation can produce even more ATP than that spent by EAATs (McKenna, 2013), however it should be noted that those experiments utilize Glu and glucose

concentrations that do not resemble physiological conditions; additionally divergent responses for cortex *versus* cerebellar astrocytes concerning metabolic functional coupling have been found (McKenna, 2012; Whitelaw and Robinson, 2013) and the use of radiolabeled substrates to follow metabolic products is not always accurate since multiple pathway interactions can not be controlled (Schousboe, 2015).

In order to set a causal relationship, a dose-response correlation between [^3H]-2DOG uptake and increasing concentrations of D-Asp and Glu was established, the EC_{50} values obtained match the K_M reported for EAAT1/GLAST-mediated Glu uptake in BGC (Ruiz and Ortega, 1995).

It is likely that after a period of intense synaptic activity, in which Glu accumulates in the extracellular space reaching concentrations in the μM range, glial Na^+ -dependent Glu transporters take up as much Glu as its K_M value enables them (approx. $26.4 \mu\text{M}$ (Ruiz and Ortega, 1995)) resulting in a Na^+ overload that activates in the one hand the Na^+/K^+ ATPase and the $\text{Na}^+/\text{Ca}^{2+}$ exchanger, resulting in a net Ca^{2+} influx (Martinez-Lozada et al., 2011), (Porras et al., 2008). Plasma membrane insertion of GLUT molecules is a rapid response largely documented, although changes in affinity of transport may explain also the increased [^3H]2DOG uptake (Ismail-Beigi, 1993). A significantly V_{Max} augmentation in BGC glucose analogue uptake with Glu treatment points to an increased number of GLUTs at the plasma membrane (Fig. 4A). A membrane biotin labelling approach sustained our hypothesis as has been documented for other preparations including astrocytes (Loaiza et al., 2003) fibroblast and mast cells (Bentley et al., 2003; Gunnink et al., 2014).

Over the past decade it has become a common feature that signal transactions triggered at the plasma membrane are the result of an activity-dependent protein-protein interactions. Molecular complexes are the key elements for an integrated cellular response. Accordingly, a presumably

physical interplay between GLUT1 and EAAT1/GLAST is inferred from co-immunoprecipitation experiments (Figure 6 A, B). This finding is in line with reports of EAAT1/GLAST interaction with the α -subunit of the Na⁺/K⁺ATPase subunit, the SNAT3, glycolytic enzymes and even mitochondria (Rose et al., 2009; Genda et al., 2011; Bauer et al., 2012; Whitelaw and Robinson, 2013).

Among the Ca²⁺-dependent kinases expressed in BGC, PKC is important for GLAST regulation (Lopez-Bayghen et al., 2003), in fact it is known to be critically involved in the transporter insertion/removal in the plasma membrane (Conradt and Stoffel, 1997) as well as in its transcriptional regulation (Lopez-Bayghen and Ortega, 2004). Therefore, it is not surprising that it participates in the signal transduction cascade that augments plasma membrane glucose transporters (Figure 5C), in fact, a specific GLUT1 S226 phosphorylation site is involved in membrane cell trafficking (Lee et al., 2015).

Of particular interest is the fact that a 30 min Glu exposure is sufficient to increase GLUT1 and GLUT3 protein levels (Figure 6, panel b). Obviously, the data can be interpreted both as an increase in GLUTs translation or a decrease in its degradation, but since Glu exposure of BGC results in a biphasic regulation of [³⁵S]methionine incorporation into TCA-precipitable polypeptides (Gonzalez-Mejia et al., 2006) it is likely that GLUTs mRNAs are regulated at the translational level by EAAT1/GLAST signaling (Barrera et al., 2008; Flores-Mendez et al., 2013).

Taken together, our results suggest that glia cells respond to neuronal Glu signaling recruiting a diverse array of proteins to the plasma membrane in a transporter-mediated manner (Fig. 7). The characterization of these signaling complexes is currently under way in our group.

Acknowledgments

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Figure legends

Fig. 1. GLUT1 y GLUT3 expression in BGC and chick cerebellum. **(a)** Total protein extracts from HeLa cells, chick BGC primary cultures and chick cerebellum were loaded equally and resolved through SDS-PAGE slab gels. A representative Western blot image of three experiments for GLUT1 and GLUT3 immunodetection is shown. The 54 kDa protein band is detected below the 66 kDa protein marker band for GLUTs. **(b)** Immunofluorescence images of GLUT1 and GLUT3 in primary BGC. GLUTs are Alexa Fluor 488 (green) immuno-labeled, TRITC (red) for Kainate Binding Protein (KBP) immunoreactive cells and nuclei are DAPI counter-stained. The scale bar represents 100 μ M. **(c)** Fifty micrometers thick sagittal sections from embrionic E18 chick cerebellum were incubated for GLUT1, GLUT3 glucose transporters, prototypical glial cells marker Kainate binding protein (KBP) and GLAST glutamate transporter. Dark precipitate from diaminobenzidine denotes positive detection of proteins, which is not evident in control slice. Purkinje cell layer is identifiable by means of calbindin staining, while the molecular layer is marked *via* GLAST and KBP, mainly GLUT1 and GLUT3 stain the thick of molecular layer in proximity to the blood brain barrier limits. Bar scale depicts ten and twenty times magnification for upper and lower panels, respectively.

Fig. 2. Glucose analogue transport in BGC is saturable with increasing time and substrate concentration. **a)** Confluent BGC monolayers were exposed at room temperature to uptake assay solution containing glucose analogue [3 H]2DOG (20 nM) plus 5 mM non-labeled 2DOG, for increasing time periods. Transport equilibrium was reached from 20 minutes incubation of substrate. **b)** Saturation curve of 2DOG transport at thirty minutes was performed with different cold 2DOG concentrations (0.1, 0.25, 0.5, 1 and 2 mM) plus a above mentioned concentration of tritiated molecule. Enzyme kinetic analyses rendered a half maximum velocity at 1.3 mM (+/- 0.4) 2DOG concentration and a Vmax of 3.1 (nmol / min / mg protein). Three independent experiments +/- SEM are graphed for each data point.

Fig. 3. Glutamate transport enhances [3 H]2DOG uptake in BGC. **(a)** Glu and its D-Asp analogue induce a 30 % increase on 2DOG uptake in BGC. Confluent monolayers were stimulated for thirty minutes with 1 mM Glu, 1 mM and 0.2 mM D-Asp, following [3 H]2DOG uptake assay for thirty minutes at room temperature was carried. **(b)** D-Asp dose-dependent stimulation of 2DOG

uptake. Increasing concentrations of D-Asp (0.01, 10, 100, 500 and 1000 μM) were incubated for thirty minutes in BGC monolayers, thereafter [^3H]2DOG uptake was performed and an EC_{50} value of 11.7 μM was estimated from a dose-response analysis. (c) Glu dose-dependent stimulation of 2DOG uptake. Increasing Glu concentrations (0.01, 10, 50, 100, 500 and 1000 μM) were incubated for thirty minutes in BGC monolayers, thereafter [^3H]2DOG uptake was performed and an EC_{50} value of 9 μM was estimated from a dose-response analysis. Results are the mean of three independent experiments \pm SEM. A one-way ANOVA identified variance significances and Dunnett's multiple comparison post-hoc test verified significant differences between control and treatment conditions in panel (a) (*** $P < 0.001$).

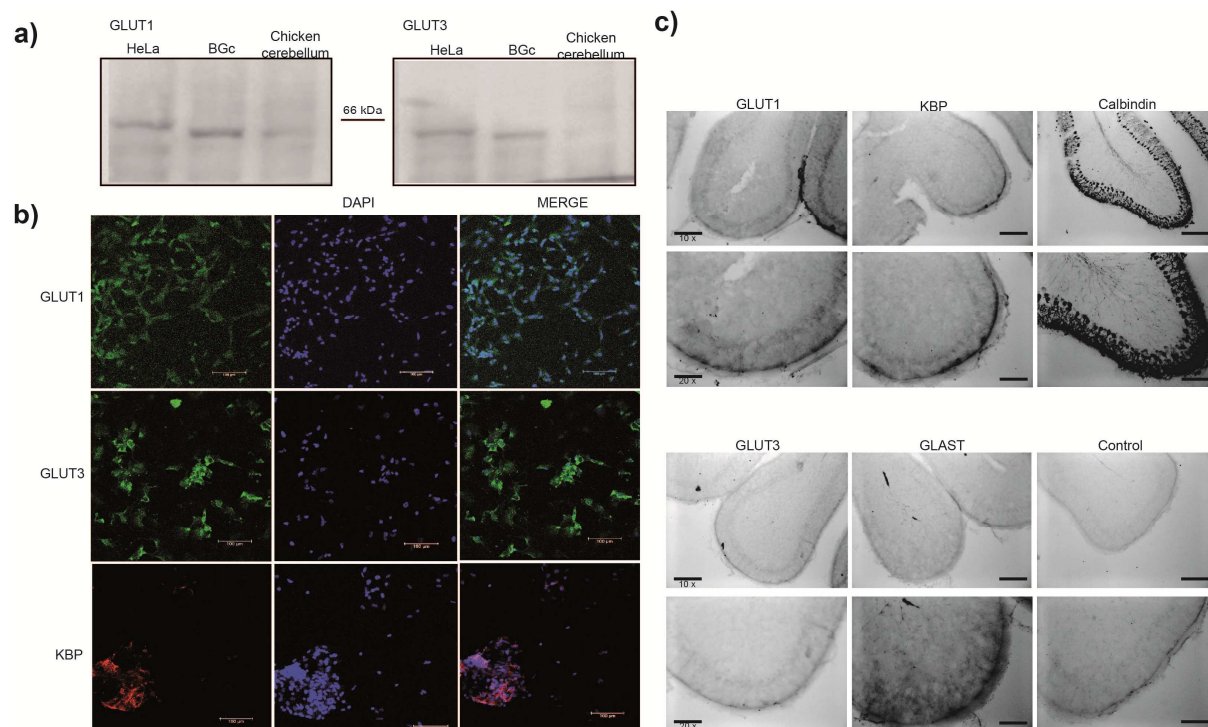
Fig. 4. Increase of maximum transport velocity and surface expression of GLUT1 in Glu stimulated BGC. (a) Kinetic analysis of glucose analogue transport in non-stimulated (NS) and 1 mM Glu treated cell monolayers. Michaelis-Menten analysis of three independent experiments were tested with a extra sum of squares F test, with a * $P < 0.05$. (b) Biotinylation of proteins from the cell surface membrane and western blot immunodetection of GLUT1 in BGC total extracts. One representative gel is shown and three independent experiments are graphed, showing extracellular membrane (biotinylated fraction) and intracellular cytoplasmic (supernatant) detection of GLUT1, with two bands around 55 kDa. One-way ANOVA analysis of average \pm SEM of three independent experiments and Dunnett's Multiple Comparison Test indicated, significant differences between NS and stimulated conditions, that are noted as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (c) Control experiment for plasmatic membrane protein biotinylation. BGC were treated as in (a), following biotinylation, agarose-streptavidin separation of proteins from total extracts and western blot for immunodetection of GLUT1 and GLAST transporters mainly from biotinylated surface membrane fraction, as well as α -actin from supernatant cytoplasmic fraction.

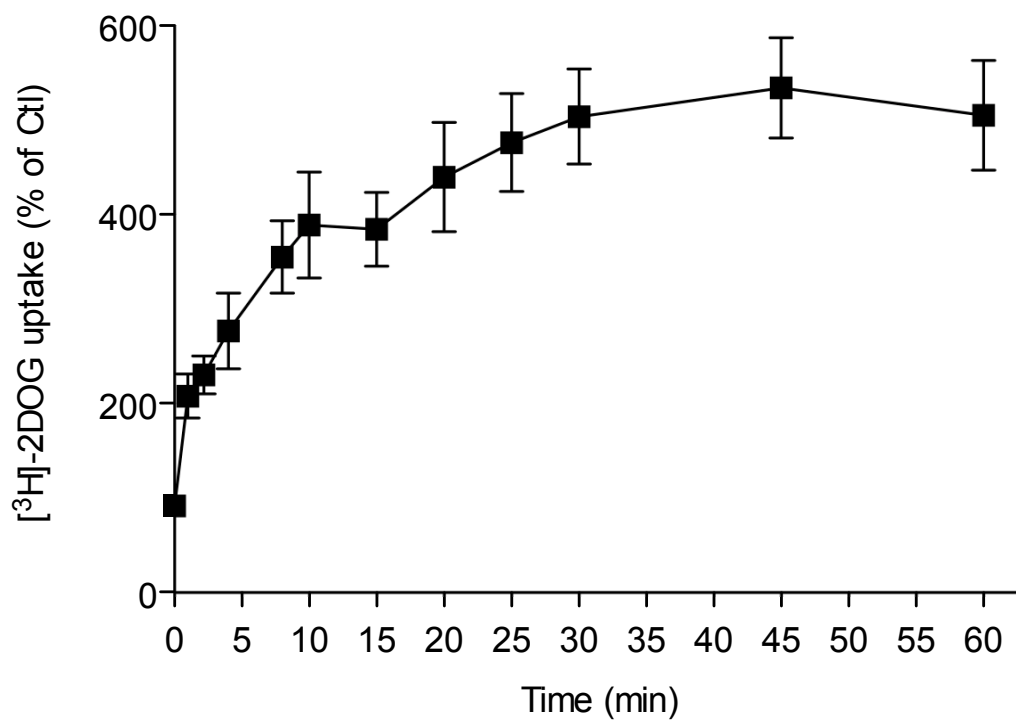
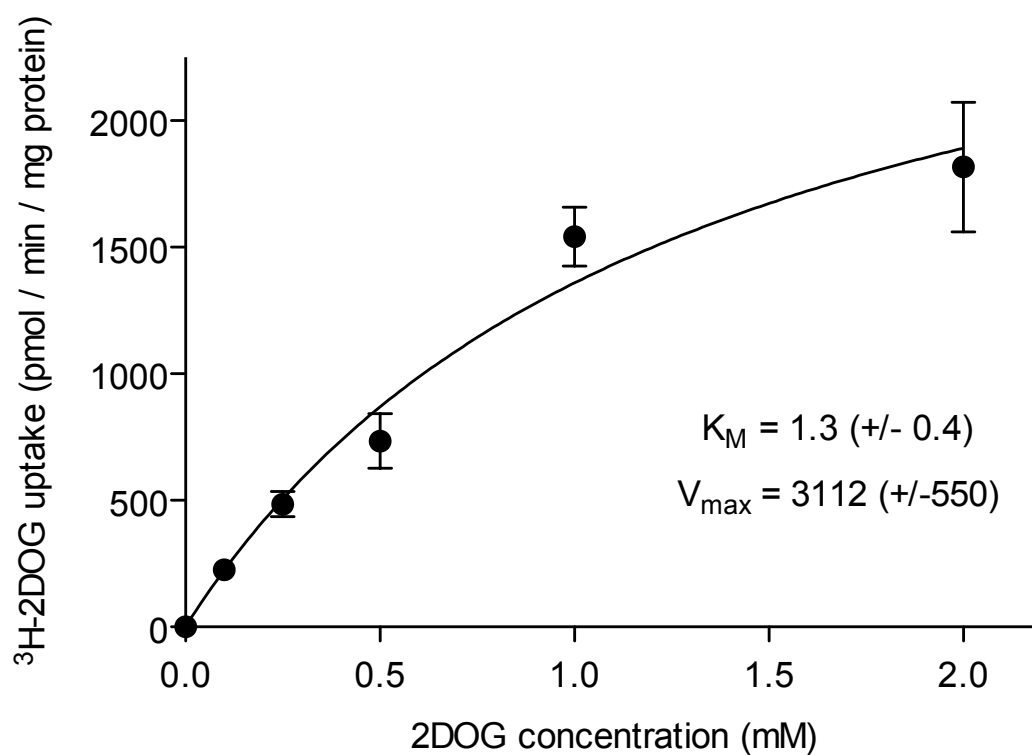
Fig. 5. Sodium and calcium dependence of Glu transport effect on [^3H]2DOG uptake increase in BGC. Thirty minutes of Glu and D-Asp treatment in complete (Na^+ and Ca^{2+}) versus sodium-free (Tris-HCl replacing NaCl) solution (a), or complete versus calcium-free (without / Ca^{2+} , plus EDTA) solution (b) followed glucose analogue uptake assay in confluent BGC monolayers. (c) Protein Kinase A (PKA), PKC and the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX) were blocked with H9 (10

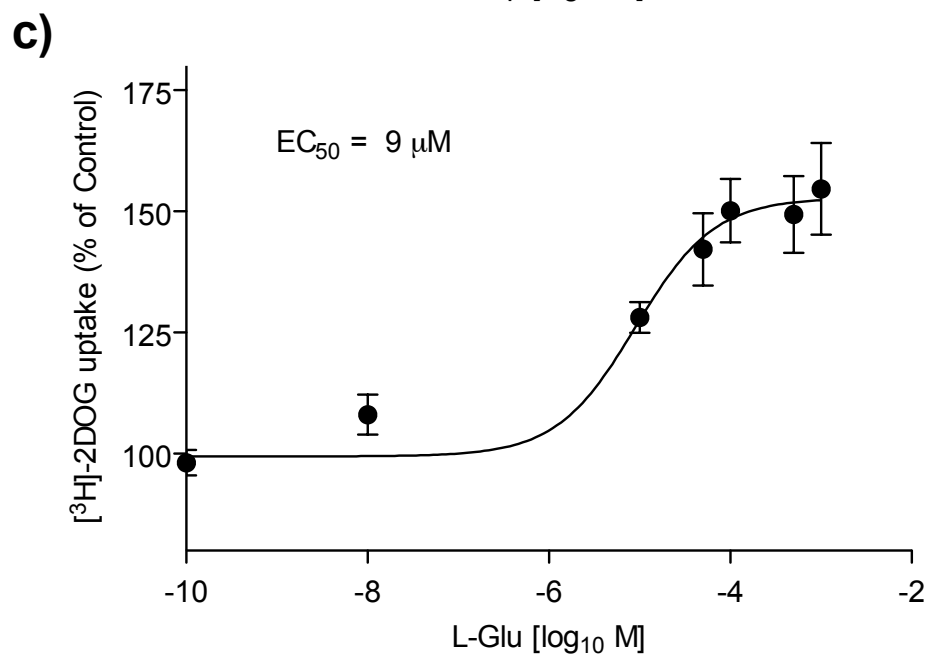
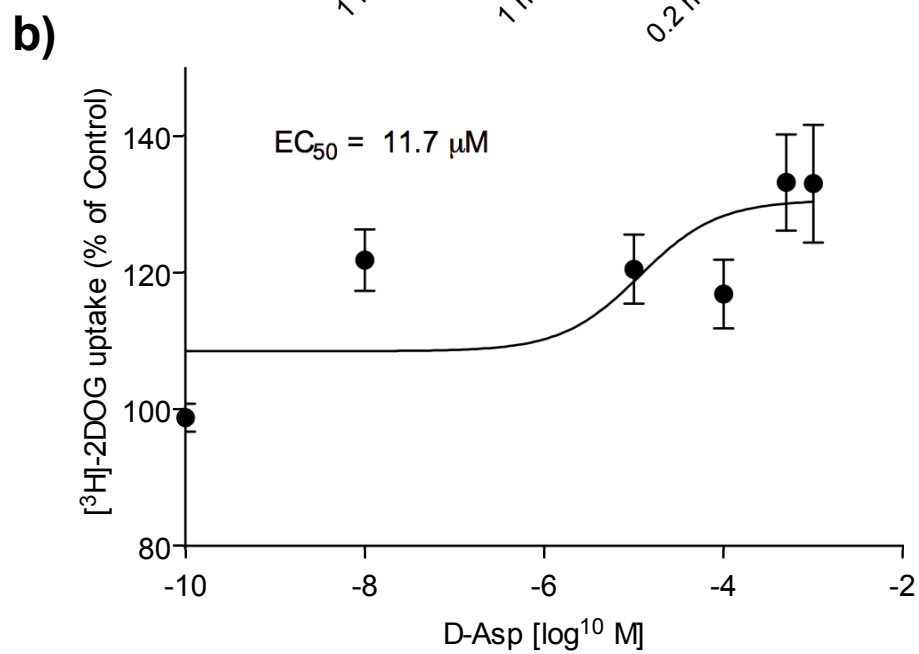
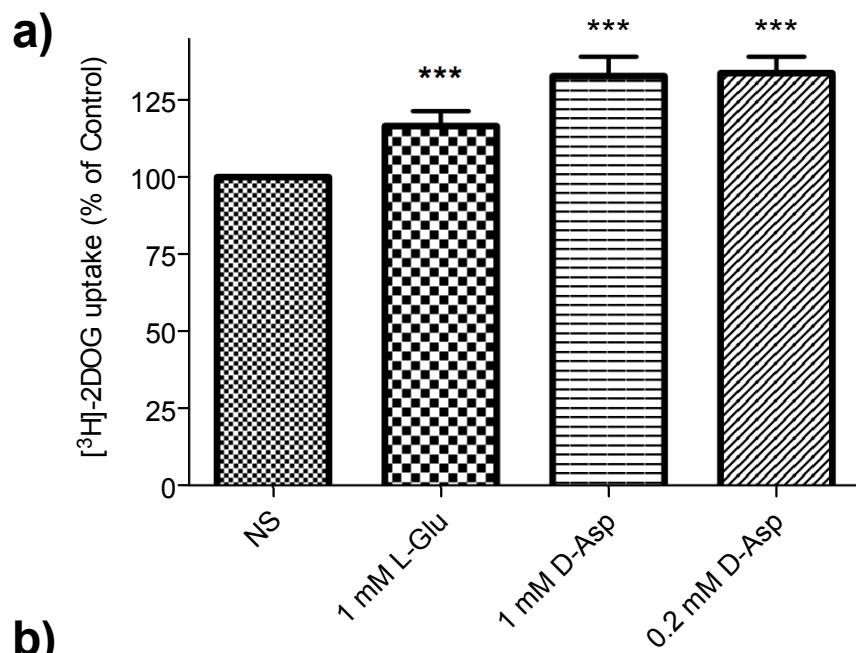
μM), Bisindolylmaleimide I (BisI, 1 μM) and KBR7943 (15 μM), respectively. **(d)** BGC monolayers were pre-exposed for 15 min to 0.1 mM TBOA (DL-threo-b-Benzoyloxyaspartic acid) and THA (threo-b-hydroxyaspartate). The results are the mean of three independent experiments \pm SEM. One-way ANOVA analysis and Dunnett's Multiple Comparison post-hoc Test were used (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

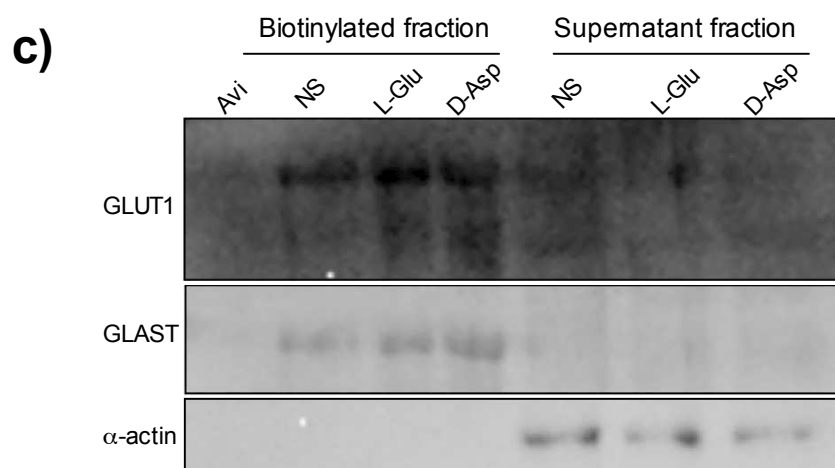
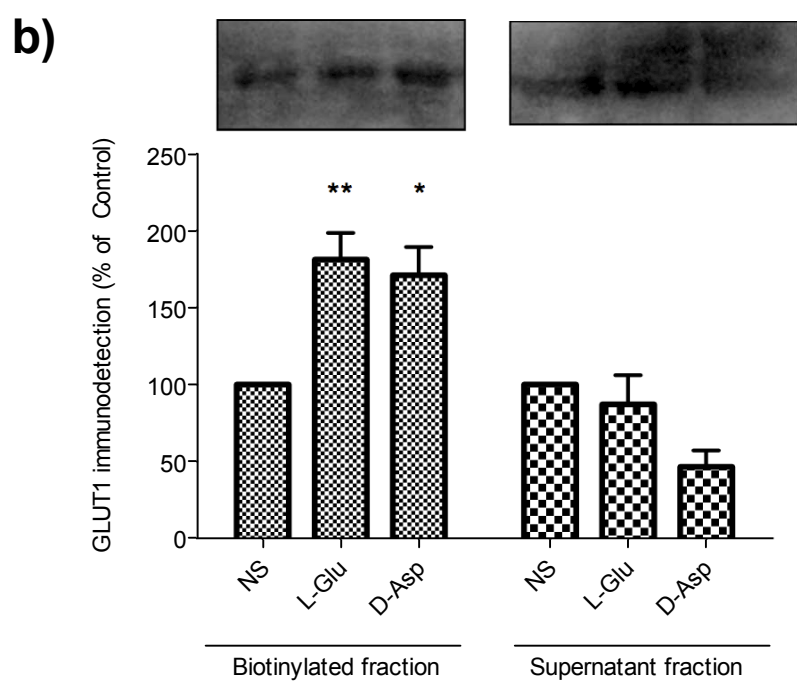
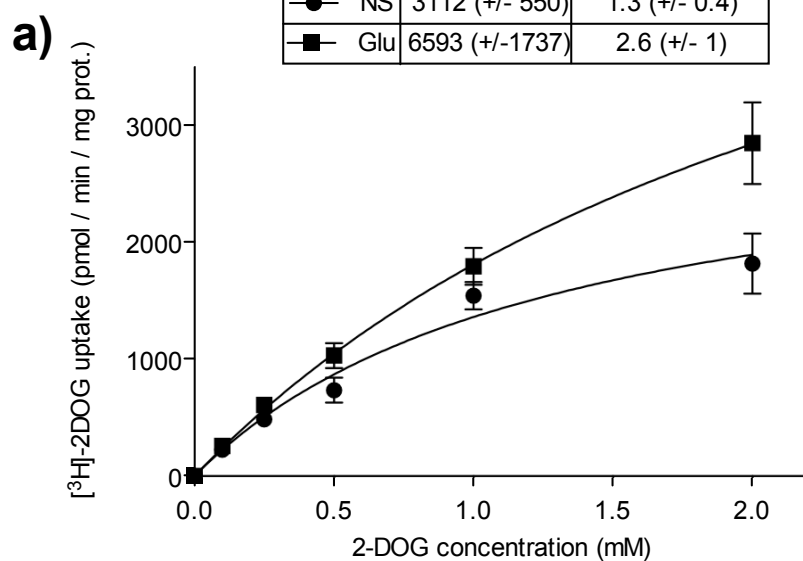
Fig. 6. Biochemical association GLUT1-GLAST. Confluent BGC monolayers were exposed to 1 mM Glu and D-Asp for 15 minutes. Cell lysates were immunoprecipitated with GLAST **(a)** and GLUT1 **(b)**, and then western blot was assayed for GLUT1 **(a)** and GLAST **(b)** immunodetection. **(c)** Equal amount of proteins from total cell lysates of 1 mM Glu and D-Asp treated BGC for 30 min were analyzed by western blot to estimate the quantity of GLUT1 and GLUT3 transporters. A representative gel image from three independent experiments is shown, bar graph represents mean value \pm SEM. One-way ANOVA analysis and Dunnett's Multiple Comparison post-hoc Test indicated significant differences in NS versus stimulated conditions (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). Further analysis for mean values of NS and Glu treatment from section **(a)** with a paired t test proved significance with a $^{##}P < 0.01$ value.

Fig. 7. Glu transport increases GLUTs membrane expression and function in BGC. From the left panel: Glu in extracellular space is taken up along with three sodium ions by high affinity glutamate transporter EAAT1/GLAST. Glu is metabolized by GS enzyme producing Gln, meanwhile ionic imbalance generated from Na^+ activates the reverse mode of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX) and the Na^+/K^+ ATPase. GS and Na^+/K^+ ATPase reduce cellular ATP. Right panel: Ca^{2+} activates PKC kinase, whose likely blank is the GLUT1 glucose transporter that associates with EAAT1/GLAST.

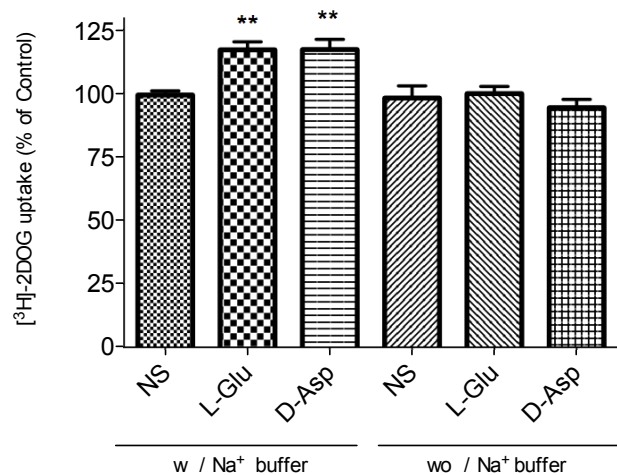


a)**b)**

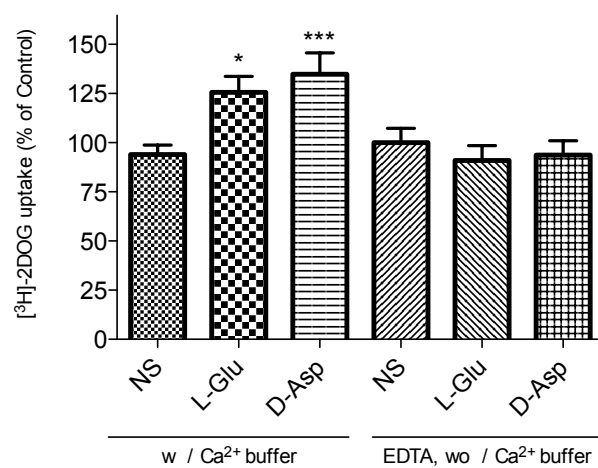




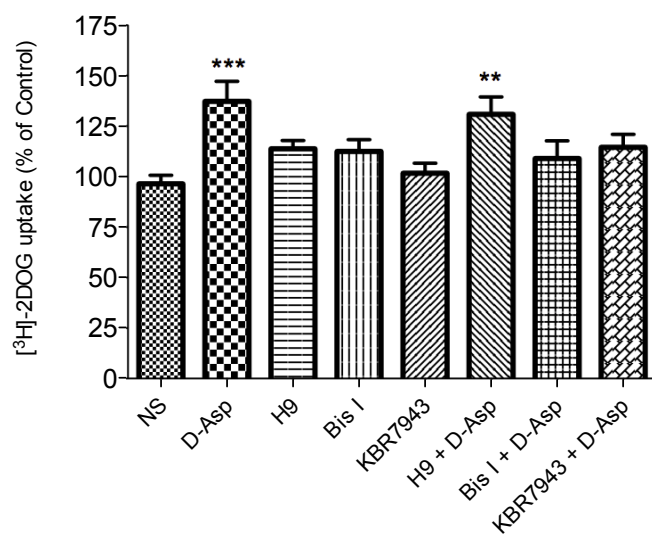
a)



b)



c)



d)

