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EAAT1-dependent *slc1a3* Transcriptional Control depends on the Substrate Translocation Process

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Abstract

Glutamate, the major excitatory neurotransmitter in the vertebrate brain, is removed from the synaptic cleft by a family of sodium-dependent transporters expressed in neurons and glial cells. The bulk of glutamate uptake activity occurs in glial cells through the sodium-dependent glutamate/aspartate transporter (EAAT1/GLAST) and glutamate transporter 1 (EAAT2/GLT-1). EAAT1/GLAST is the predominant transporter within the cerebellum. It is highly enriched in Bergmann glial cells that span the cerebellar cortex and wrap the most abundant glutamatergic synapses in the central nervous system, the synapse formed by the parallel fibers and the Purkinje cells. In the past years, it has become evident that Bergmann glial cells are involved in glutamatergic transmission. Glutamate transporters are tightly regulated due to their essential role in tripartite synapses. Glutamate regulates EAAT1/GLAST function and gene expression in a receptor-dependent and receptor-independent manner. Through the use of the non-metabolizable EAAT1/GLAST ligand, D-Aspartate, and the well-established chick cerebellar Bergmann glia primary culture, in this contribution, we demonstrate that EAAT1/GLAST down-regulates its expression and function at the transcriptional level through the activation of a signaling pathway that includes the phosphatidyl inositol 3 kinase (PI3K), the Ca^{2+}/diacylglycerol dependent protein kinase PKC and the nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB). These results favor the notion of an activity-dependent fine-tuning of glutamate recycling and its synaptic transactions through glial cells.

Summary statement

EAAT1/GLAST down-regulates its expression and function at the transcriptional level by activating a signaling pathway that includes PI3K, PKC and NF-κB, favoring the notion of an activity-dependent fine-tuning of glutamate recycling and its synaptic transactions through glial cells.

Keywords

Bergmann glia, glutamate transporters, transcriptional control

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Introduction

Glutamate (Glu) mediates most of the brain's fast excitatory synaptic transmission by activating specific plasma membrane receptors and transporters. Two main subtypes of Glu receptors have been characterized: ionotropic and metabotropic. These receptors' pharmacological and molecular properties have been reviewed (Reiner & Levitz, 2018). Ionotropic receptors are ligand-gated anionic channels named after their specific agonists: N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and kainate (KA).

Overstimulation of these receptors has been associated with neuronal and oligodendrocyte cell death, a phenomenon

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known as excitotoxicity. It has long been suggested that excitotoxic insults are related to neurodegenerative disorders, such as amyotrophic lateral sclerosis, multiple sclerosis, Parkinson's, and Alzheimer's disease (Lau & Tymianski, 2010; O'Donovan et al., 2017).

A family of sodium-dependent Glu transporters, collectively known as Excitatory Amino Acid Transporters (EAATs), regulate Glu extracellular levels, maintaining the concentrations of this amino acid between 1 and 3 μM and protecting cells against cytotoxicity (Danbolt, 1994; Lucas & Newhouse, 1957). Five subtypes of Glu transporters (EAAT1-5) have been characterized. Most of the Glu uptake occurs in glial cells through the sodium-dependent Glu/aspartate transporter (EAAT1/GLAST) and Glu transporter 1 (EAAT2/GLT-1) (Gonzalez & Ortega, 1997; Marcaggi & Attwell, 2004; Rothstein et al., 1996; Tzingounis & Wadiche, 2007). Glu transport is coupled to the cotransport of three Na^+ ions and one proton into the cell, while one K^+ ion is transported out (Zerangue & Kavanaugh, 1996). Moreover, EAATs also function as anion-selective channels (Machtens et al., 2015).

Cerebellar Bergmann glial cells (BGC) are radial glia that does not differentiate into astrocytes after birth. These cells function as a neuronal reservoir and represent more than 90% of the cerebellar glial cells (Cameron & Rakic, 1991). BGCs are involved in neurotransmitter uptake, K^+ homeostasis, and pH regulation and express a battery of receptors and transporters that enable them to sense neuronal activity (Eulenburg & Gomeza, 2010; Lopez-Bayghen et al., 2007; Lopez-Colome et al., 2012; Slotboom et al., 1999). EAAT1/GLAST mediates the vast majority of Glu uptake activity within the cerebellum in BGC (Danbolt et al., 1998; O'Donovan et al., 2017; Rothstein et al., 1994). Glial cells directly influence neuronal and synaptic actions by releasing neuroactive substances such as cytokines, neuromodulators, and neurotransmitters like Glu and glycine that affect synaptic development, maintenance, and recovery (Angulo et al., 2004; Auld & Robitaille, 2003).

The reduced expression of Glu transporters impacts the extracellular concentrations of this amino acid, favoring frequent seizures and neuronal degeneration. These events link glial cells and the development of some neurological pathologies (Auld & Robitaille, 2003). The number and distribution of glial Glu receptors and transporters influence the strength and dynamics of glutamatergic neurotransmission. A well-studied regulator of the expression and activity of Glu transporters is the Ca^{2+}/diacylglycerol-dependent protein kinase (PKC). Eleven PKC isoforms encoded by nine genes have been described and grouped into three classes: classical, novel, and atypical, that, upon activation, trigger differential effects on Glu transport (Gonzalez & Robinson, 2004; Vandenberg & Ryan, 2013). In the case of cerebellar glial cells, PKC activation decreases the uptake activity through a reduction in V_{Max} that results from a diminished transporter cell surface expression that might be linked to a transcriptional down-regulation (Gonzalez & Ortega, 1997; Vandenberg & Ryan, 2013).

The molecular mechanisms responsible for EAAT1/GLAST decreased activity in BGC have been characterized and involve the Yin Yang 1 (YY1) transcription factor. Glu exposure leads to a receptor-mediated PKC-dependent YY1 binding to the *slc1a3* promoter, halting its transcription, diminishing mRNA and protein levels and consequently, Glu uptake (Rosas et al., 2007).

Another study has reported that NF-κB is a critical positive EAAT1/GLAST regulator (Karki et al., 2015). On the other hand, Glu activates a wide variety of signal transduction cascades related to Glu transporter turnover in the plasma membrane. For example, it has been documented that nitric oxide regulates EAAT1/GLAST by activating guanylate cyclase and the cGMP-dependent protein kinase (PKG) in a Ca^{2+} dependent manner (Balderas et al., 2014). Another group reported a reduction in Glu uptake after inhibiting the calcium/calmodulin-dependent kinase (CaMKII) in HEK293T2 and cortical astrocytic cultures transfected with EAAT1/GLAST (Chawla et al., 2017).

Despite these studies, the signaling pathways that participate in EAAT1/GLAST regulation are not entirely understood. Our group and others demonstrated that Glu and some of its analogs, including D-aspartate (D-Asp), down-regulate EAAT1/GLAST activity (Danbolt et al., 1998; Gonzalez & Ortega, 2000). Using the established model of chick cerebellar BGC cultures , we characterized the D-Asp effect on EAAT1/GLAST regulation and examined the possible signaling pathways involved in this process.

Materials and Methods

Reagents

Tissue culture reagents were obtained from Life Technologies (Carlsbad, CA, USA). D-Asp, phorbol 12-myristate 13-acetate (TPA), L-trans-Pyrrolidine-2,4-dicarboxylic acid (PDC), Actinomycin D (Act-D), and dimethyl sulfoxide (DMSO) were purchased to Sigma-Aldrich (St. Louis, MO, USA). DL-threo-β-Benzyloxy aspartic acid (DL-TBOA), 2-[2-[4-(4-Nitrobenzyloxy)phenyl]ethyl] isothioureamesylate (KB-R7943), N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W7) were all obtained from Tocris-Cookson (St. Louis, MO, USA). [^{3}H]-D-Asp (Specific activity 16.5 Ci/mmol, 1 mCi/mL) was obtained from Perkin Elmer (Boston, MA, USA). TRIzol reagent was obtained from Invitrogen (Waltham, MA, USA). Wortmannin, Bisindolylmaleimide I (BisI), and PKC inhibitor 20-28 were purchased from Calbiochem (San Diego, CA, USA). Caffeic acid was from Abcam (Boston, USA).

Cell Culture and Stimulation Protocol

Primary cultures of cerebellar BGCs were prepared from a pool of cerebellum from 14-day-old chick embryos with

subtle adjustments from our previously described protocol (Ortega et al., 1991). Cells (7×10^5 cells) were seeded in 24 or 48-well plates (Corning, NY) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, and 50 mg/ml gentamicin at 37 °C under standard conditions (5% CO_2 and 95% humidity) and used on the fourth day after culture. Before any treatment, confluent monolayers were switched to low-serum DMEM media (0.5% FBS) for two hours, then D-Asp was applied (0.15–1000 μM); In the case of signaling pathway analysis, specific inhibitors were added 30 min before D-Asp treatment (all initially dissolved in DMSO). Experiments were carried out in triplicates, and reported results are from at least three independent experiments.

Glutamate Transporter Assays in BGC

BGC monolayers, seeded in 48-well plates, were treated with D-Asp at the indicated concentrations and time periods. After treatment, [^{3}H]-D-Asp uptake, used as a non-metabolizable analog of L-Glu, was performed at room temperature (25 °C) as detailed by Ruiz & Ortega, 1995 (Ruiz & Ortega, 1995). Briefly, the culture medium was exchanged for Solution A (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, 1 mM Na_2HPO_4, pH 7.4) and the cells were preincubated for 30 min. Immediately, the medium was exchanged with Solution A containing [^{3}H]-D-Asp (0.4 mCi/ml) and incubated for 30 min. Rapid media aspiration stopped the uptake, and the monolayers were washed with ice-cold Solution A and solubilized with 0.1M NaOH. The Bradford method was used with one aliquot of the sample for protein determination by the Bradford method, and the sample's radioactivity was determined by liquid scintillation counting in a Perkin Elmer B291001 scintillation counter. For saturation experiments, different concentrations of unlabeled D-Asp were added, and the kinetic constants (V_{Max} and K_M) were determined with a non-linear regression analysis (Prism, GraphPad).

RNA Extraction and Quantitative RT-PCR (qRT-PCR)

Confluent BGC cultures were treated with D-Asp and/or inhibitors (PDC, TBOA, Bis I, PKCI 20–28, KB-R7943, W7, or Caffeic acid) as indicated in each Figure. Total RNA was isolated using TRIzol Reagent and the Directzol MiniPrep kit (Zymo Research, CA, USA). Each treatment condition per experiment was analyzed in duplicate. Quantitative qRT-PCR was performed with the One-step NZY Speedy RT-qPCR Green kit, ROX plus (NZYtech, Lisbon, Portugal) in a reaction volume of 10 μl [20 ng of total RNA, Green master mix ROX plus (1x), NZYRT mix, forward primer (200 nM) and reverse primer (200 nM)], using Step One Plus Real-time PCR System (Applied Biosystems). The reaction consisted of a cDNA synthesis step at 50°C for 20 min followed by RT inactivation at 95° C for 5 min. After that, 40 cycles at 95°C for 5 s and the specific annealing temperature (T_a) for each primer pair for 30 s. Melting curves capillary electrophoretic analyses were constructed to determine the purity and verify if the bands corresponded with theoretical melting temperatures. To quantify mRNA levels, we used previously designed and reported oligonucleotides:

slc3a1 Forward 5′- GGCTGCGGGCATTCCTC-3′
slc3a1 Reverse 5′-CGGAGACGATCCAAGAACCA-3′

As an endogenous control, we amplified the ribosomal protein S17 mRNA with the following primers: S17 Forward 5′-CCGCTGGATGCGCTTCATCAG-3′

S17 Reverse 5′-′TACACCCGTCTGGGCAAC-3′ (Lopez et al., 2019).

The relative abundance of each mRNA is expressed as sample versus control compared to control normalized to S17 chick ribosomal mRNA and was calculated using the $2^{-\Delta\Delta}$Ct method.

SDS-PAGE and Western Blots

Confluent BGC cultures were treated with D-Asp for different time periods. The confluent monolayers were harvested, and 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). Equal amounts of total protein extracts (approximately 50 μg as determined by the Bradford method) were denatured in Laemmli's sample buffer and resolved through 10% SDS polyacrylamide gels and electroblotted onto nitrocellulose membranes. Membranes were incubated in PBS containing 5% dried skimmed milk and 0.1% Tween 20 for two hours to block the excess of non-specific protein-binding sites. Then, the membranes were incubated overnight at 4°C with the primary antibodies (anti-GLAST antibody) (RRIDAB_2915901) (Martinez-Lozada et al., 2013) and anti-actin monoclonal antibodies (Diaz-Barriga et al., 1989) diluted in 5% dried skimmed milk and 0.1% Tween in TBS buffer, followed by secondary antibodies (Anti-Mouse, RRID: AB_2533947; Anti-Rabbit, RRID AB_87750). Immuno-reactive polypeptides were detected by chemiluminescence with MicroChemi (DNR Bio-Imaging System). Densitometry analyses were performed with the Image J application.

Transient Transfections and Luciferase Assays

BGC seeded into 24-well plates were transfected with 150 ng of NF-κB-Luc using Lipofectamine™ 3000 (Invitrogen, CA, USA). The reporter plasmid pGL3Luc-5XNF-kappaB was constructed with double-strand oligonucleotides that contain

five repeats of NF-κB binding consensus sequence (5′-GGGGAATTTCC-3′) (Wong et al., 2011), cloned in the pGL3Luc-promoter vector (Promega, Madison, USA). Briefly, the plasmids were mixed with Optimem, p300 reagent, and Lipofectamine 3000. The mix was incubated for 10 min at room temperature, and the cells were incubated with this mixture for 24 h. Then, the cells were treated with caffeic acid with or without D-Asp for a further 24 h. Cells were subsequently harvested and lysed with 150 µl of 1X reporter lysis buffer (Promega), and protein lysates were obtained by two freeze-thawing cycles. Luciferase assays were performed with the Luciferase Assay System (Promega) with equal amounts of the protein lysates. Detection was performed with an Infinite M200 PRO plate reader (TECAN, Männedorf, Switzerland).

Statistical Analysis

All data were analyzed using the Prism 6 GraphPad Software (San Diego, CA, USA; RRID: SCR_002798). Data are expressed as mean ± Standard Error of the Mean (SEM). A one-way analysis of variance (ANOVA) was performed to determine significant differences between groups. When this analysis indicated significance (>0.05 level), a *post hoc* Bonferroni multiple comparison analysis was used to determine which groups were significantly different. Statistical significance is represented as follow: $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. References were handled with EndNote Software (RRID: SCR_014001)

Results

D-Asp Inhibits GLAST Uptake Activity

The effect of D-Asp exposure on GLAST transport activity was evaluated. To this end, primary cultures of BGCs were exposed to D-Asp, and EAAT1/GLAST transport activity was assessed by [^{3}H]-D-Asp uptake assays. EAAT1/GLAST transport activity decreased in cells treated with concentrations ranging from 1.5 to 1000 µM of D-Asp for 24 h (Figure 1A). Treatments with 150 µM of D-Asp for shorter times also decreased transporter activity. A 3-h exposure resulted in a significant 20% activity reduction ($p < 0.001$; Figure 1B). Kinetic analysis of the transporter activity showed that D-Asp decreased V_{Max} and slightly increased K_M, favoring the idea of a D-Asp-dependent reduction in functional EAAAT1/GLAST transporters in the plasma membrane (Figure 1C).

D-Asp Treatment Downregulates slc1a3 Transcription

The reduction in active EAAT1/GLAST transporters in the plasma membrane due to D-Asp pre-treatment could be explained in several manners; one possibility could be a reduction in *slc1a3* mRNA levels. Therefore, a qRT-PCR strategy was undertaken. As depicted in Figure 2A, D-Asp treatment resulted in a 40% decrease in *slc1a3* mRNA levels after the exposure to 150 and 1000 µM concentrations ($p < 0.01$), which correlates with a reduction of EAAT1/GLAST protein levels (Figure 2b). These results strongly suggest that the transcription of the gene encoding EAAT1/GLAST (*slc1a3*) might be modulated by D-Asp treatment. To rule out *slc1a3* mediated mRNA degradation, we measured *slc1a3* mRNA half-life. As depicted in panel (c) of Figure 2, D-Asp does not modify the half-life of *slc1a3* mRNA (Figure 2C).

PI3K/PKC Signaling Pathway is Involved in the D-Asp-Dependent slc1a3 Transcriptional Regulation

The signaling properties of EAAT1/GLAST have been described in radial glial cells (Martinez-Lozada & Ortega, 2015). Na^+ entry through the transporter triggers the reverse function of the Na^+/Ca^{2+} exchanger, increasing $[Ca^{2+}]_i$. We have previously established a role for PKC in the transcriptional regulation of *slc1a3* (Lopez-Bayghen & Ortega, 2004; López-Bayghen et al., 2003). Therefore, we decided to explore the involvement of PKC in the D-Asp-dependent *slc1a3* transcriptional control. When BGC monolayers were treated with PDC, a transportable EAAT1/GLAST blocker, a similar response to that observed with D-Asp was present. In contrast, a non-transportable blocker like TBOA does not reproduce the D-Asp effect as no significant difference was recorded compared to the non-treated control (Figure 3A). Interestingly, the general PKC inhibitor, BisI, and the specific PKC alpha inhibitor, PKCI 20-28, significantly prevented the downregulation of *slc1a3* mRNA levels caused by D-Asp treatment ($p < 0.01$, Figure 3B). These results suggest that PKC alpha is involved in *slc1a3* transcription regulation. Accordingly, when BGC cultures were treated with the PKC activator TPA, a 50% decrease in *slc1a3* mRNA levels was recorded ($p < 0.001$, Figure 3c). Since *slc1a3* mRNA levels are downregulated by D-Asp, *via* PKC, more specifically PKC alpha, it could be expected that a calmodulin (CaM) antagonist such as W-7 would not block the D-Asp effect as this is not a Ca^{2+}/CaM -dependent pathway. Indeed, this is the case. Interestingly, when the BGC cultures were pre-exposed to the Na^+/Ca^{2+} exchanger blocker KB-R7943, no inhibition of the D-Asp effect was observed (Figure 4A); This is an unexpecyed result for which we do not have an explanation. In contrast, the PI3K inhibitor wortmannin blocks the D-Asp-dependent reduction in *slc1a3* mRNA levels (Figure 4b). These results indicate that PKC alpha is a downstream effector of PI3K.

NF-KappaB Downregulates slc1a3 Expression

Since an *in-silico* analysis showed that the *slc1a3* promoter contains at least two NF-κB binding sites, which are critical for regulating this promoter (Karki et al., 2015; Unger et al., 2012), we

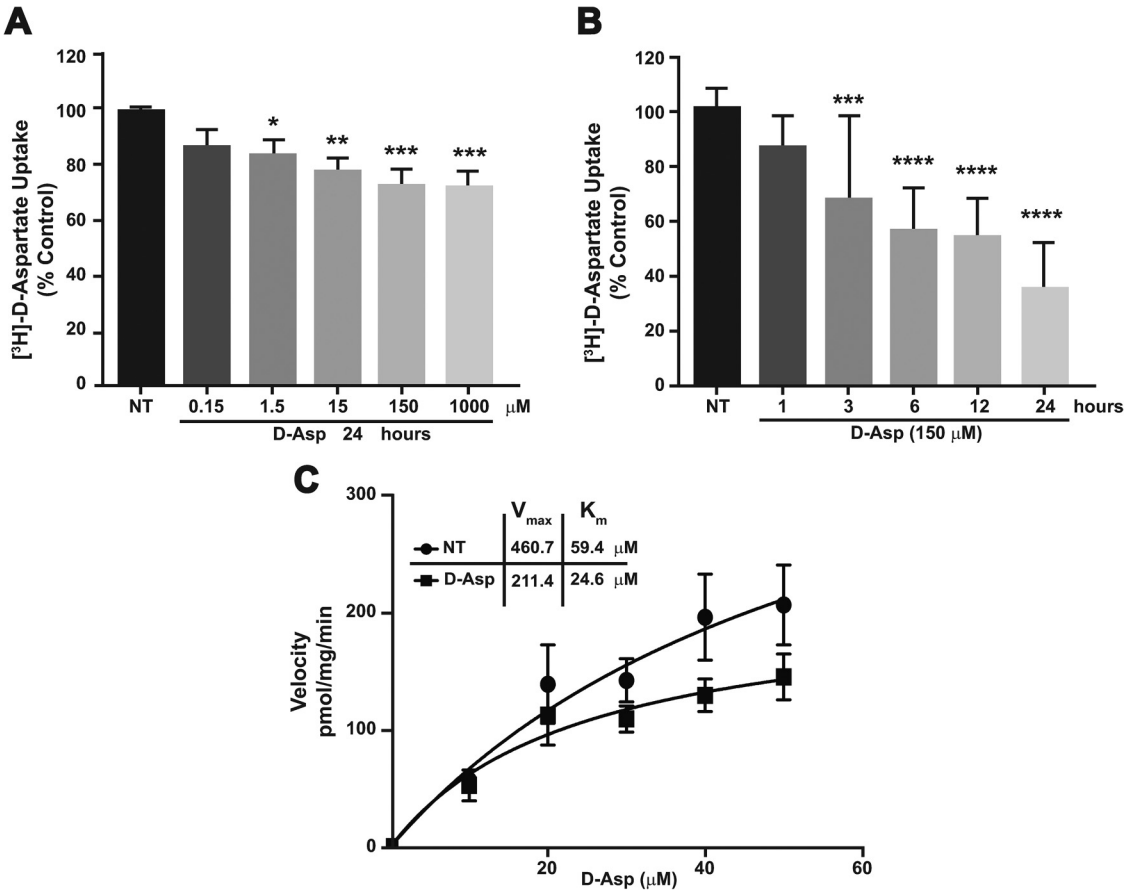

Figure 1. Long-term exposure to D-Asp impairs GLAST activity in BGC. Confluent BGC primary cultures were treated with D-Asp, and GLAST activity was measured by[[^{3H}]-D-Asp (0.4 mCi/mL) uptake. (A) The dose-dependent effect was analyzed when BGC cultured cells were incubated at 0.15, 1.5, 15, 150, and 1000 μM with D-Asp for 24 h. (B) BGCs were treated with 150 μM D-Asp for 1, 3, 6, 12, and 24 h to determine the time-dependent effect. (C) The kinetics effects of D-Asp on the Na^+-dependent [^{3H}]-D-Asp transport were assessed when BGC cultures were pre-incubated with 150 μM D-Asp (squares) or non-treatment (circles) for 12 h; the kinetic constants were determined with non-linear regression analysis. All results were normalized to protein content and are presented as a percentage of control (NT). Each bar is the mean value ± SEM in all three panels from three independent experiments performed in quadruplicates. One-way ANOVA with a *post hoc* Bonferroni test was performed. *p <0.05, **p <0.01, ***p < 0.001, and ****p < 0.0001.

decided to explore if NF-κB participates in *slc1a3* transcriptional regulation by D-Asp. When BGC cultures were pretreated with caffeic acid, a specific NF-κB inhibitor, the D-Asp was effectively prevented ($p < 0.001$, Figure 5a). Moreover, when a luciferase reporter vector containing the NF-κB consensus sites (pNF-κB-Luc construct) was transfected into BGCs, D-Asp treatment resulted in an approximately 50% increase in promoter activity, which, as expected, is sensitive to caffeic acid ($p < 0.05$, Figure 5b), pointing out that NF-κB is involved in the D-Asp-triggered membrane to nuclei cascade that reduces EAAT1/GLAST protein levels disrupting BGC Glu uptake capacity. A known PKC activator, TPA, was used as a positive control of NF-κB activation.

Discussion

The results presented in this contribution demonstrate that EAAT1/GLAST activity, protein, and *slc1a3* mRNA levels diminish in a time- and dose-dependent manner upon D-Asp exposure. Furthermore, previous results from our group, reported by Gonzalez et al., demonstrated that EAAT1/GLAST transportable blockers mimicked a Glu-dependent reduction of the uptake activity. Interestingly enough, the effect was sensitive to a serine/threonine-protein kinase inhibitors such as staurosporine (Gonzalez & Ortega, 2000).

The actual Glu concentrations in the synaptic cleft of after periods of high activity have been calculated in the high micromolar or even low millimolar range in different preparations. In fact, under basal circumstances, [Glu] lies in the 0.2 to 5 μM range [for a review (Rodriguez-Campuzano & Ortega, 2021)]. Moreover since the EC_{50} of most ionotropic Glu receptors and transporters lies in the micromolar range, it is quite possible that high glutamatergic activity periods trigger the described *slc1A3* transcriptional repression (Figure 5). It then might be argued that the time course of such high Glu concentrations in the synaptic cleft, takes

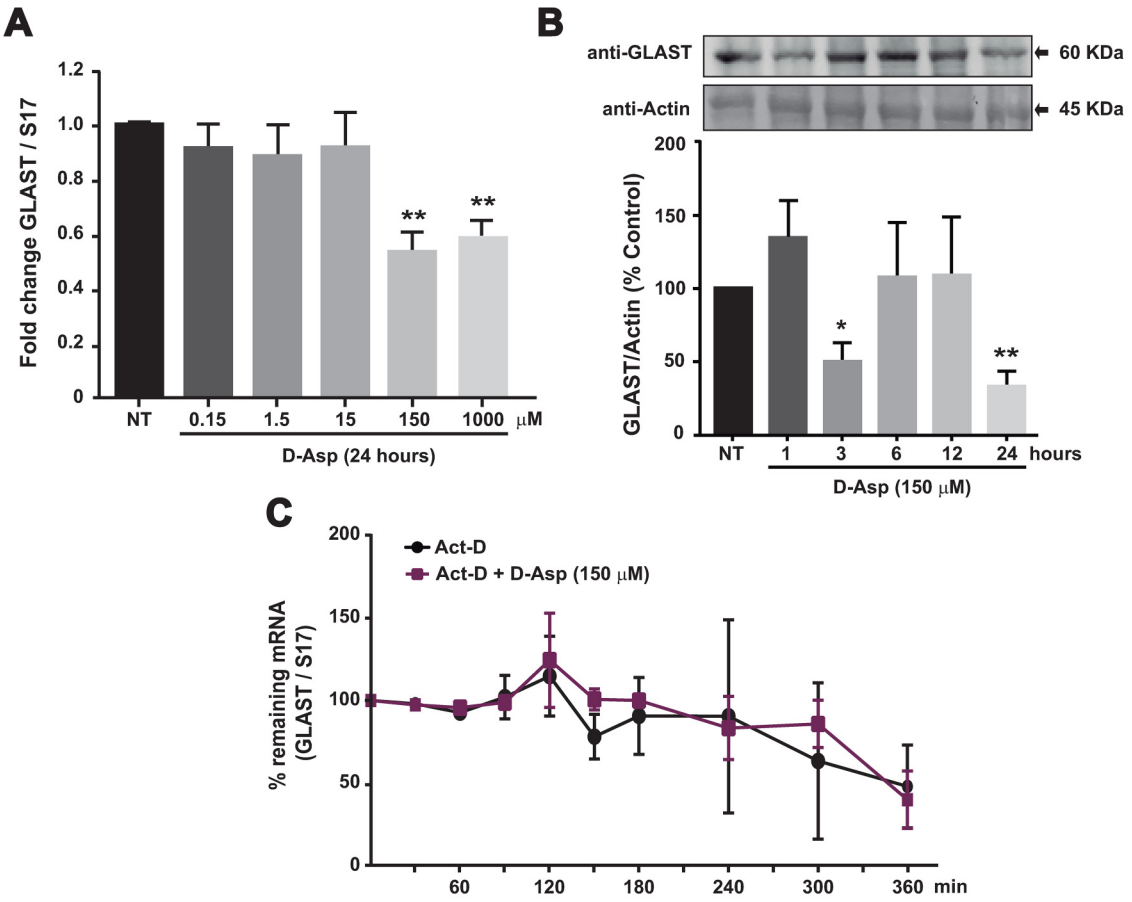

Figure 2. D-Asp downregulates EAAT1/GLAST expression in BGC. Confluent BGC cultures were treated with D-Asp, and *slc1a3* expression was analyzed. (A) BGC cultures were incubated with D-Asp at 0.15, 1.5, 15, 150, and 1000 μM for 24 h. Afterward, total RNA was extracted, and *slc1a3* mRNA levels were quantified by qRT-PCR. Data were normalized to NT. Each bar is the mean ± SEM from three independent experiments, performed in triplicate and normalized using S17. (B) To determine EAAT1/GLAST protein levels by Western Blot with anti-EAAT1/GLAST in a total protein extract of BGC treated with 150 μM of D-Asp for 0, 1, 3, 6, 12, and 24 h. Actin was used as a loading control. Data are expressed as the mean ± SEM of EAAT1/GLAST/Actin normalized to NT for three independent experiments. A representative blot is presented is shown. (C) D-Asp effects on *slc1a3* mRNA stability were determined when BGC cultures were treated with DMSO (Control) or 4 μM Act-D (a transcriptional inhibitor) alone or with 150 μM D-Asp. Cells were harvested every 30 min up to 360 min. Total RNA was extracted, and *slc1a3* mRNA levels were quantified by qRT-PCR. Data were normalized at DMSO (control) to time = 0. Each bar is the mean ± SEM from three independent experiments, performed in triplicate and normalized using *S17*. One-way ANOVA followed by a post hoc Bonferroni test was performed. *p <0.05, and **p <0.01.

place in the milliseconds to seconds time frame, which is expected in a physiological scenario. Nevertheless, we have shown in previous publications, that the long Asp treatments (hours) are not needed for EAAT1/GLAST signaling (Martinez-Lozada et al., 2011, 2013., 2014), reviewed in (Olivares-Banuelos et al., 2019). In fact, the acute effect of a 30 min 1 mM Glu exposure on the [^{3}H] D-Asp uptake activity in BGC lasts for three hours (Martinez-Lozada & Ortega, 2015). In this context, it is clear that *slc1a3* transcriptional down-regulation does not necessarily need a prolonged D-Asp exposure; as previously shown by our group, a 30 min L-Glu reduces the transcription of this gene (Rosas et al., 2007). It is clear that our results point out that the transporter signaling regulates glial physiology in the short, medium, and long term.

The reduction in EAAT1/GLAST transporter activity upon D-Asp treatment (Figure 1) could occur by two possible mechanisms: 1) a decrease in the number of transporters on the cell surface, or 2) a significant reduction of the affinity of the transporter protein for its substrate. Therefore, we performed a Michaelis-Menten analysis to gain insight into the molecular mechanism involved in the effect. As a result, we found that the reduced uptake after D-Asp treatment is related mainly to a decrease in the maximum velocity of the transport (V_{Max}) since its affinity displayed a discrete increase (K_M). Previously, Gonzalez and Ortega demonstrated that the PKC activator, TPA, diminishes the transporter's V_{Max}, suggesting a decrease in the number of transporters on the BGC plasma membrane (Gonzalez & Ortega, 1997). A

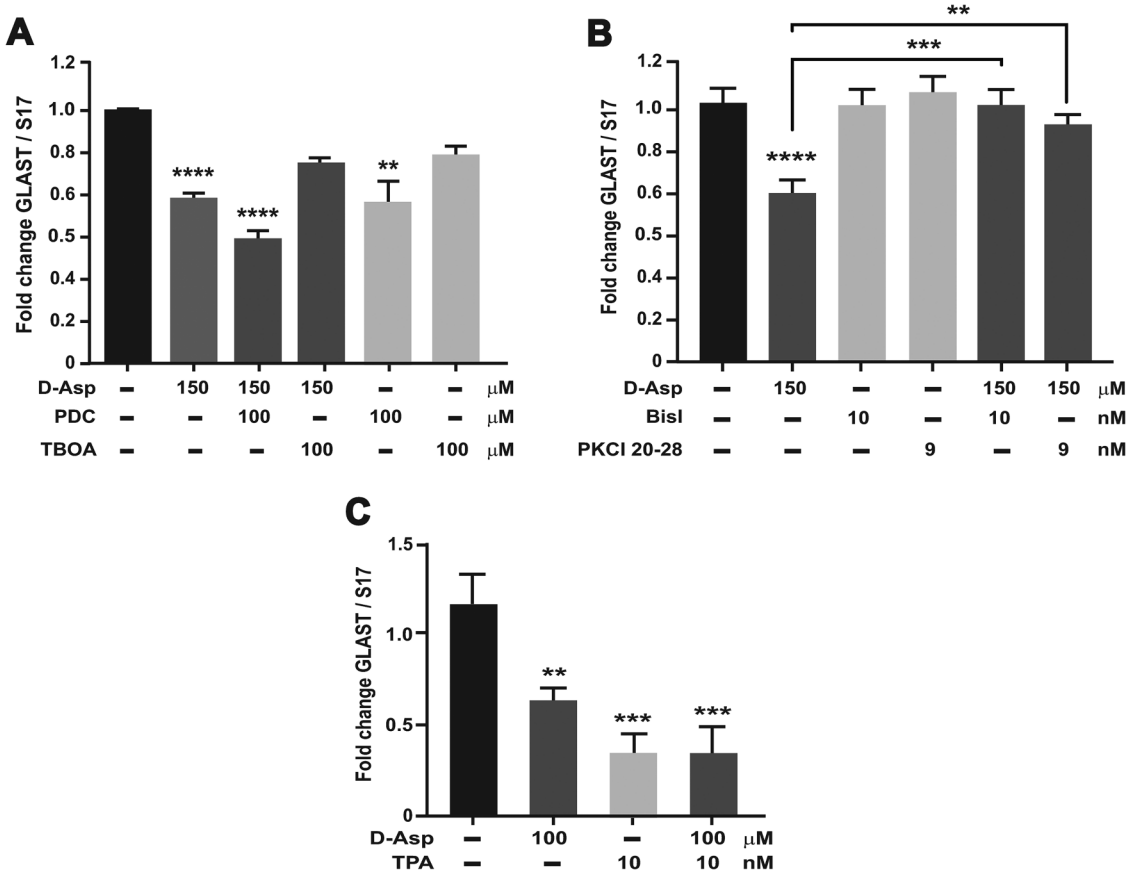

Figure 3. PKC-alpha involvement in D-As -dependent *slc1a3* transcriptional downregulation confluent BGC cultures were pretreated with (A) 100 μM PDC (trans-4-Carboxy-L-proline, transportable glutamate inhibitor) or 100 μM TBOA (DL-threo-β-Benzyloxyaspartic acid, a selective non-transportable inhibitor of EAATs), (B) PKC inhibitors: 10 nm Bisl or 8 μm protein kinase c inhibitor 20–28, or (C) 10 nm TPA (Tetradecanoylphorbol-13-acetate, PKC activator) for 30 min, followed by either 100 μM or 150 μM D-Asp or DMSO (used as control; inhibitor vehicle in a 0.1% final concentration) for 24 h. Total RNA was isolated, and *slc1a3* expression was assessed via qRT-PCR. Data were normalized to control. Each bar is the mean ± SEM from three independent experiments, performed in triplicate and normalized using *S17*. One-way ANOVA followed by a *post hoc* Bonferroni test was performed to determine. $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

similar effect was reported in retina glial Müller cells by Wang *et al*. (Wang et al., 2003).

These results prompted us to evaluate *slc1a3* mRNA and EAAT1/GLAST protein levels to rule out an effect in the insertion of transporters in the plasma membrane (Rodriguez-Campuzano & Ortega, 2021). A clear reduction in EAAT1/GLAST activity, protein levels, and *slc1a3* mRNA is present after D-Asp long-term (> 3 h) treatment. Moreover, *slc1a3* mRNA half-life remains unaltered, strongly suggesting a transcriptional repression effect like the one described for the PKC activator, TPA (Espinoza-Rojo et al., 2000).

It has been documented that PKC is indirectly involved in EAAT1/GLAST activity regulation since mutation of its putative phosphorylation sites does not hamper the TPA-dependent decrease in Glu uptake (Bull & Barnett, 2002). Furthermore, several receptors expressed in glial cells are linked to the activation of the various PKC isoforms in the cerebellum, specifically in BGC; Glu is known to activate PKC in a receptor-dependent and independent manner (Cid & Ortega, 1993; Lopez-Bayghen & Ortega, 2004; Martinez-Lozada et al., 2011). In fact, Glu treatment regulates its uptake by two PKC-dependent mechanisms: 1) in the short-term (min), Glu reduces EAAT1/GLAST transporter protein at the cell surface in a receptor-independent fashion, and 2) in the long-term, exposure triggers a transcriptional regulation of *slc1a3* gene through the activation of Ca^{2+}-permeable AMPA receptors (Espinoza-Rojo et al., 2000; Ortega et al., 1991).

In this contribution, we decided to evaluate an alleged involvement of the transport process in the *slc1a3* mRNA expression and long-term regulation, since Na^+ entry triggers (*via* the reverse function of the Na^+/Ca^{2+} exchanger) several signaling cascades (Martinez-Lozada & Ortega, 2015). The results presented here show that *slc1a3* transcriptional downregulation by the specific transporter substrate D-Asp, involves a PI3K/PKC alpha pathway that does not include the Na^+/Ca^{2+} exchanger since preincubation with KB-R7943 does not block the D-Asp reduction in

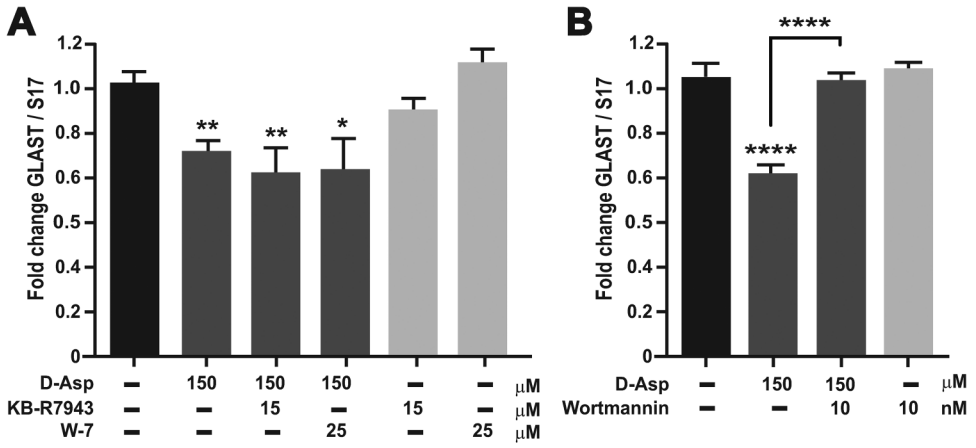

Figure 4. Involvement of PI3K but not of the Na^+/Ca^{2+} exchanger or CaM in the D-Asp dependent transcriptional down regulation. Confluent BGC cultures were pretreated with Na^+/Ca^{2+} exchange inhibitor, KB-R7943 (15 μM) and calmodulin antagonit W7 (25 μM) in (A), or specific PI3K inhibitor, Wortmannin (10 nM) and DMSO (control; inhibitor vehicle in a 0.1% final concentration) in (B). Then, BGCs were treated with D-Asp (150 μM) for 24h. Total RNA was extracted, and *slc1a3* mRNA levels were measured by qRT-PCR. Data were normalized to control (DMSO treated), and each bar represents the mean ± SEM from three independent experiments, performed at least in triplicate and normalized using *S17*. A one-way ANOVA was performed to determine whether there were significant differences between groups with the Bonferroni post hoc test. *p <0.05, **p < 0.01 and ****p < 0.0001.

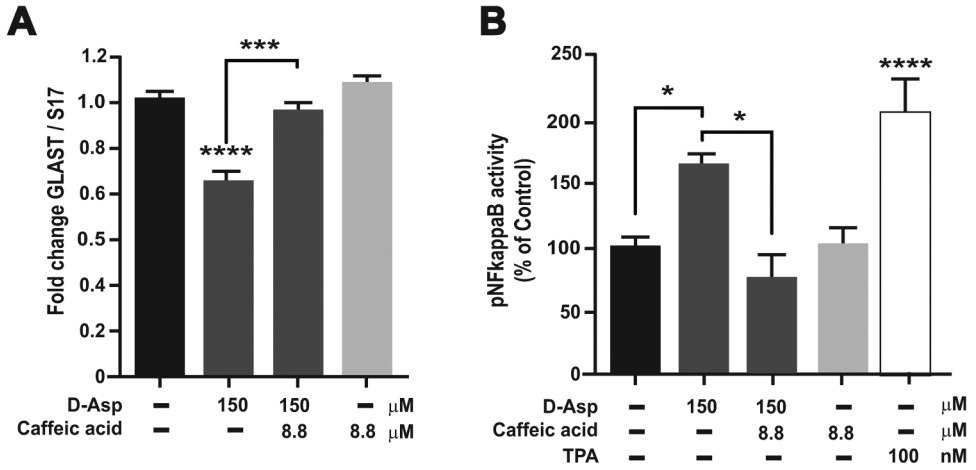

Figure 5. *Slc1a3* expression is downregulated by NF-κB. Confluent BGC cultures were either pretreated with 8.8 μM caffeic acid (specific NF-κB inhibitor), or DMSO, for 30 min before being treated with 150 μM D-Asp or DMSO for 24 h (used as control; inhibitor vehicle in a 0.1% final concentration). (A) Total RNA was extracted, and *slc1a3* mRNA levels were measured by qRT-PCR. Data were normalized to control, and each bar represents the mean ± SEM from three independent experiments, performed in triplicate and normalized using *S17*. (B) pNFκB-Luc construct, containing the repeated consensus sequence (6x), was transfected into BGCs with Lipofectamine 3000. Cell lysates were analyzed using the luciferase assay to measure promoter activity. Data were normalized to the control. The values are mean ± SEM in all panels from at least three individual experiments performed in triplicate. One-way ANOVA followed by a *post hoc* Bonferroni test was performed. ***p < 0.001, and ****p < 0.0001.

slc1a3 mRNA levels. A CaMKII signaling cascade can be discarted since CaM inhibition with W7 fails to prevent the D-Asp effect. (Figure 4). In agreement, the overexpression of a classical (alpha) or a novel PKC isoform (epsilon) in BGC downregulated the *slc1a3* promoter activity as well as uptake (Lopez-Bayghen & Ortega, 2004). A similar effect was observed in retina glial Müller cells. Glu transport was significantly decreased in cells exposed to the PKC activator PMA, and this effect was confirmed by blocking it with the specific PKC inhibitor, BisI. Furthermore, PKC reduces plasma membrane EAAT1/GLAST and its uptake activity (Bull & Barnett, 2002; Wang et al., 2003).

Glu transporter expression is regulated at the transcriptional, translational, and post-translational levels (Gegelashvili &

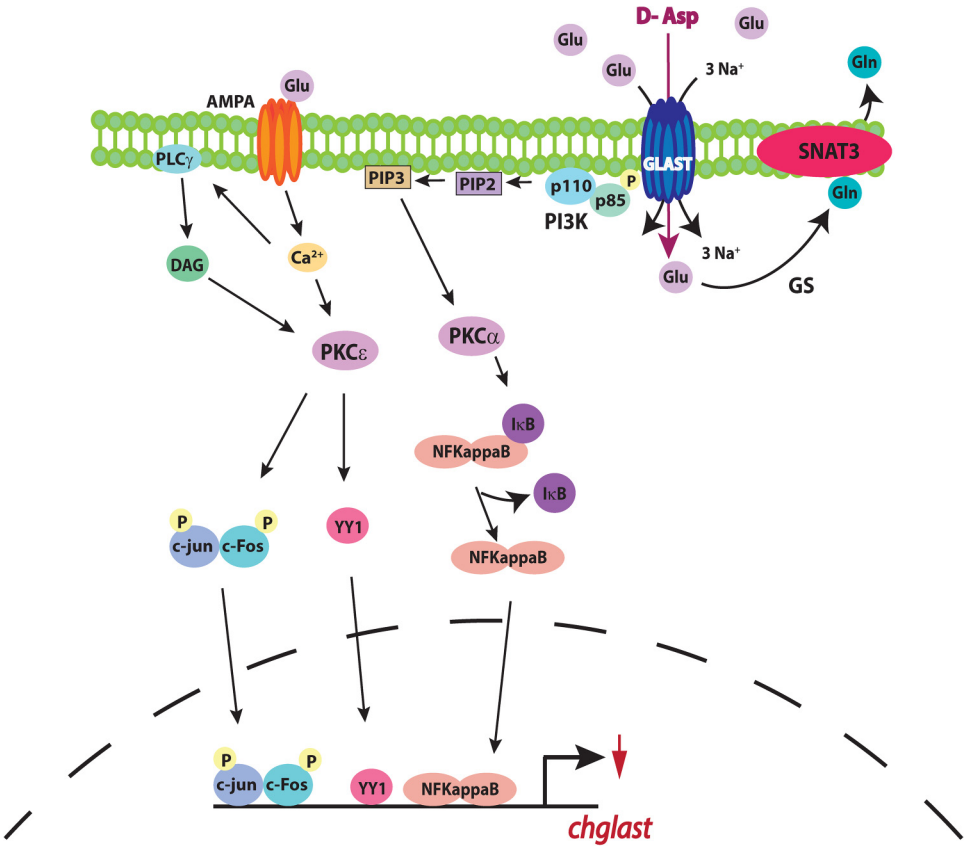

Figure 6. AMPA receptors and GLAST activities regulate *slc1a3* transcription: comparison of signaling events in BGC. AMPA receptors trigger Ca^{2+} activation; consequently, PKC (epsilon or alpha) downregulates *slc1a3* transcription by promoting the AP-1 and YY-1 nuclear localization. On the left side, GLAST transporter activity is involved in the negative transcriptional regulation. Glu uptake leads to PI3K activation that activates PKC alpha to repress *slc1a3* expression *via* NF-κB.

Schousboe, 1997; Martinez-Lozada et al., 2016) and reviewed in (Rodriguez-Campuzano & Ortega, 2021). Several reports have shown that different stimuli regulate *slc1a3* gene transcription. Dibutyryl cAMP treatment increased *slc1a3* mRNA and protein levels in primary cultures of rat astrocytes (Guillet et al., 2005; Swanson et al., 1997). Glial cell line-derived neurotrophic factor (GDNF), insulin-like growth factor-1 (IGF-1), basic fibroblast growth factor (bFGF), and epidermal growth factor (EGF) increase *slc1a3* mRNA and protein levels.

The results described confirm that the transport process (D-Asp treatment) regulates EAAT1/GLAST both at the short and long term. The *slc1a3* promoter is highly conserved between species, it contains a GC box that is recognized by the transcription factors stimulating proteins 1 and 3 (Sp1 and Sp3), activator protein-1 (Ap-1), and YY-1, and also contains two *bonna fide* NF-κB consensus sequence (Hagiwara et al., 1996; Kim et al., 2003; López-Bayghen et al., 2003; Stoffel et al., 1996).

Therefore, we explored NF-κB's role over *slc1a3* transcriptional regulation. Our results show that D-Asp treatment down-regulates *slc1a3* transcription *via* NF-κB (Figure 5). Caffeic acid (CA), and its synthesized derivative, caffeic acid phenethyl ester (CAPE), are selective transcriptional down-regulators by inhibiting NF-kappaB. Activation of NF-kappa B by tumor necrosis factor (TNF) is completely blocked by CAPE in a dose- and time-dependent manner as it prevents the translocation of the p65 subunit of NF-kappa B to the nucleus, specifically inhibiting NF-kappa B binding to the DNA (but not affecting the binding of AP-1 for example) (Chung et al., 2004; Natarajan et al., 1996; Watabe et al., 2004). Also, NF-κB exerts transcriptional control *via* PKC in other systems (Moscat et al., 2003). In contrast, it has been described that NF-κB up-regulates *slc1a3* expression *in vitro* in an ischemic model after treatment with group-II metabotropic Glu receptors (mGluR2/3) agonist (Lin et al., 2014). Also, Tai et al. confirmed that amitriptyline/morphine co-infusion induced EAAT1/GLAST upregulation, increases IkBα phosphorylation and the translocation of NF-κB p65 from the cytosol to the nucleus (Tai et al., 2008). Nevertheless, Rosas et al. reported that YY-1 decreased *slc1a3* transcriptional response to Glu in BGC. Furthermore, Glu induces (through AMPA receptors and PKC) an increase in YY1-DNA binding, which reduces *slc1a3* promoter activity and mRNA levels (Rosas et al., 2007). Moreover, it was found

that Manganese repressed *slc1a3* by activating NF-κB, and co-expression of both NF-κB and YY-1 completely suppressed the stimulatory NF-κB effect, suggesting that YY1 is dominant over NF-κB p65 (Karki et al., 2015).

In summary, we provide here compelling evidence for the direct involvement of EAAT1/GLAST transporter activity in its own (*slc1a3)* transcriptional regulation through a PI3K/PKCα/NF-κB signaling pathway, which is different from the AMPA receptors pathway described previously (Figure 6). A pertinent question raises about the activation of either cascade *in vivo* by Glu. It is tempting to speculate that the transporter-dependent path might be the primary *slc1a3* transcriptional regulator since Glu transporters outnumber Glu receptors in glial cells (reviewed by (Rodriguez-Campuzano & Ortega, 2021)). Work currently in progress in our labs aims to characterize the NF-κB role in *slc1a3* down-regulation.

Author Contribution

D.H.M. and L.R.M. contributed equally and must be considered the first authors. Methodology, formal analysis, investigation, writing—original draft preparation D.H.M. and L.R.M.; methodology, data analysis C.P.G. and L.C.C.; funding acquisition, supervision, visualization, writing-review and editing ELB and AO; conceptualization and final manuscript AO. All authors have read and agreed to the published version of the manuscript.

Declaration of Conflicting Interests

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Abbreviations

KB-R7943	2-[2-[4-(4-Nitrobenzyloxy)phenyl]ethyl] isothioureamesylate
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
Act-D	actinomycin D
Ap-1	activator protein-1
BGC	Bergmann glial cells
BisI	bisindolylmaleimide I
PKC	Ca^{2+}/diacylglycerol-dependent protein kinase
CaMKII	calcium/calmodulin-dependent kinase
PKG	cGMP-dependent protein kinase
DMSO	dimethyl sulfoxide
DL-TBOA	DL-threo-β-Benzyloxy aspartic acid
DMEM	Dulbecco's modified Eagle's medium
EAATs	Excitatory Amino Acid Transporters
Glu	glutamate
GLT-1	glutamate transporter 1
KA	kainate
PDC	L-trans-Pyrrolidine-2,4-dicarboxylic acid
W7	N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride
NMDA	N-methyl-D-aspartate
NF-κB	nuclear factor κ-light-chain-enhancer of activated B cells
TPA	phorbol 12-myristate 13-acetate
YY1	quantitative *RT-PCR (qRT-PCR),* Yin Yang 1