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Signaling through EAAT-1/GLAST in cultured Bergmann glia cells

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ABSTRACT

Glutamate, the major excitatory amino acid, activates a wide variety of signal transduction cascades. Synaptic plasticity relies on activity-dependent differential protein expression. Ionotropic and metabotropic glutamate receptors have been critically involved in long-term synaptic changes, although recent findings suggest that the electrogenic Na^+-dependent glutamate transporters, responsible of its removal from the synaptic cleft, participate in glutamate-induced signaling. Transporter proteins are expressed in neurons and glia cells albeit most of the glutamate uptake occurs in the glial compartment. Within the cerebellum, Bergmann glial cells are close to glutamatergic synapses and participate actively in the recycling of glutamate through the glutamate/glutamine shuttle. In this context, we decided to investigate a plausible role of Bergmann glia glutamate transporters as signaling entities. To this end, primary cultures of chick cerebellar Bergmann glial cells were exposed to D-aspartate (D-Asp) and other transporter ligands and the serine 2448 phosphorylation pattern of the master regulator of protein synthesis, namely the mammalian target of rapamycin (mTOR), determined. An increase in mTOR phosphorylation and activity was detected. The signaling cascade included Ca^{2+} influx, activation of the phosphatidylinositol 3-kinase and protein kinase B. Furthermore, transporter signaling resulted also in an increase in activator protein-1 (AP-1) binding to DNA and the up-regulation of the transcription of an AP-1 driven gene construct. These results add a novel mediator of the glutamate effects at the translational and transcriptional levels and further strengthen the notion of the critical involvement of glia cells in synaptic function.
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1. Introduction

Excitatory neurotransmission in the vertebrate central nervous system (CNS) is mediated largely by glutamate (Glu). Two main subtypes of Glu receptors have been defined: ionotropic (iGluRs) and metabotropic receptors (mGluRs). Three iGluRs exist: *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoaxazolepropionate (AMPA) and kainate (KA) receptors (Hollmann and Heinemann, 1994). Metabotropic receptors are divided in terms of sequence similarity, signal transduction mechanisms and pharmacology in three groups. Group I receptors are coupled to the stimulation of phospholipase C with the consequent release of intracellular Ca^{2+}, while Groups II and III are coupled to the inhibition of adenylate cyclase. These three groups are activated preferentially by (RS)-3,5-dihydroxyphenylglycine (DHPG) for Group I, (S)-4-carboxy-3-hydroxyphenylglycine (S)-4C3HPG activates Group II while L-(+)-2-amino-4-phosphonobutyric acid (L-AP4) acts upon Group III (Coutinho and Knopfel, 2002).

Bergmann glia cells (BGCs) are the most abundant glia cells in the cerebellum, comprising more than 90% of the cerebellar glia. These cells span the entire cerebellar molecular layer and encapsulate neuronal somata, dendrites and axons. BGC are involved in neurotransmitter uptake, K^+ homeostasis and pH regulation due to the expression of a battery of receptors and transporters (Lopez-Bayghen et al., 2007). In terms of glutamatergic transmission, BGC are in a very short proximity to the parallel fiber-Purkinje cell synapses, and are involved in the Glu/glutamine shuttle that assures the Glu supply to the presynaptic terminals. In this sense, BGC respond to glutamatergic stimulation, as we have been able to characterize over the years (Barrera et al., 2010).

Activity-dependent gene expression regulation stabilizes the synaptic changes that underlie the late phase of long-term potentiation (Pittenger and Kandel, 1998). Transcription and translation are essential for long-term memory (Hu et al., 2006). While most studies have focused in gene expression regulation at the transcriptional level, regulation of protein synthesis has a crucial role in synaptic plasticity (Cammalleri et al., 2003). Translational

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control offers the possibility of a rapid response to external stimulus without mRNA synthesis and transport. Therefore, immediacy is the most conspicuous advantage of translational over transcriptional control.

The mammalian target of rapamycin (mTOR) is a master regulator of protein synthesis (Proud, 2007). It is a multi-domain serine/threonine kinase that phosphorylates a wide array of proteins like phosphatase 2A and Huntingtin. It forms the catalytic core of two different complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). During acute exposure, rapamycin inhibits mTORC1 but not mTORC2. In a rather simplified scenario, mTORC1 mediates the mTOR effects that are rapamycin-sensitive. The canonical pathway that leads to mTOR serine 2448 phosphorylation and thus activation, includes phosphatidylinositol 3-kinase (PI3-K), which produces phosphatidylinositol 3,4,5-triphosphate (PIP3), that anchors phosphoinositide-dependent kinase 1 (PDK1) and protein kinase B (PKB) to the cell membrane (Sabatini, 2006). PKB is activated through a sequential phosphorylation cascade by PDK1 and a PDK2 activity that now it has been shown to correspond to mTORC2 (Bayascas and Alessi, 2005). Phosphorylated PKB activates mTORC1 that acts upon several translation components like eukaryotic initiation factor 4E binding protein-1 (4EBP1) and the 70 kDa S6 ribosomal kinase ($p70^{S6K}$) increasing protein synthesis (Bayascas and Alessi, 2005; Foster and Fingar, 2010).

Glu is removed from the synaptic cleft by a family of electrogenic sodium-dependent transporters expressed in neurons and glia cells (Danbolt, 2001). Five subtypes of transporters named excitatory amino acids transporters 1–5 (EAAT-1–5) have been characterized. The glial transporters EAAT-1 (GLAST) and EAAT-2 (GLT-1) account for more than 80% of the Glu uptake activity in the brain (Eulenburg and Gomeza, 2010; Swanson, 2005). Within BGC, EAAT-1/GLAST is the predominant transporter (Maragakis et al., 2004). Evidences suggest that Glu transporters might also participate in the signaling transactions triggered by this amino acid. In fact, Glu regulates the uptake process in a receptor-independent manner (Gonzalez and Ortega, 2000). More recently, it has also been reported that EAAT-1 is coupled to the Na^+/K^+ ATPase (Gegelashvili et al., 2007; Rose et al., 2009). To provide further evidence for a role of EAAT-1/GLAST in Glu signaling, in the present contribution we challenged the plausible participation of Glu transporters in gene expression regulation. We show here that Glu uptake is linked to an increase in the translation process and that it is also coupled to the transcriptional activation of an AP-1 driven construct. These results are discussed in terms of the physiological significance of an alternative signaling entity to Glu receptors and the identity of the genes regulated. A preliminary description of a D-Asp-dependent mTOR phosphorylation was reported earlier (Zepeda et al., 2009).

2. Materials and methods

2.1. Materials

Tissue culture reagents were obtained from GE Healthcare (Carlsbad, CA, USA). A23187 (5-(methylamino)-2-({(2*R*,3*R*,6*S*,8*S*,9*R*,11*R*)-3,9,11-trimethyl-8-[(1*S*)-1-methyl-2-oxo-2-(1*H*-pyrrol-2-yl)ethyl]-1,7-dioxaspiro[5.5] undec-2-yl}methyl)-1,3-benzoxazole-4-carboxylic acid), Wortmannin, Amiloride (3,5-diamino-6-chloro-*N*-(diaminomethylene)pyrazine-2-carboxamide), KB-R7943 (2-[2-[4-(4-Nitrobenzyloxy)phenyl]ethyl]isothioureamesylate), DL-TBOA (DL-threo-β-Benzyloxyaspartic acid), THA (threo β–hydroxyaspartate), DNQX (6,7-Dinitroquinoxaline-2,3-dione), LAP5 (L-(+)-2-Amino-5-phosphonopentanoic acid); CPCCOEt (7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxylate ethyl ester), CPPG ((RS)-α-cyclopropyl-4-phosphonophenylglycine), PP2 (4-amino-5-(4-chlorophenyl)-7-(dimethylethyl)pyrazolo[3,4-*d*]pyrimidine, genistein and D-aspartate (D-Asp) and Glu were all obtained from Tocris-Cookson (St. Louis, MO, USA). PDC (L-trans-Pyrrolidine-2,4-dicarboxylic acid) was purchased to Sigma–Aldrich (St. Louis, MO, USA); ^{45}Ca was from Perkin Elmer (Boston, MA, USA). Polyclonal anti-phospho-mTOR (Ser 2448) and anti mTOR antibodies (05–235) were purchased from Cell Signaling Technology (Beverly, MA, USA). Polyclonal phospho-4EBP1 (Thr 70) was purchased from Santa Cruz Biotech, (Santa Cruz, CA, USA, sc-18092-R). Monoclonal anti-actin antibodies were kindly donated by Prof. Manuel Hernández (Cinvestav-IPN). Horseradish peroxidase-linked anti-rabbit and anti-mouse antibodies, and the enhanced chemiluminescence reagent (ECL), were obtained from Amersham Biosciences (Buckinghamshire, UK). 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 as previously described (Ortega et al., 1991). Cells were plated in 6 or 24-well plastic culture dishes in DMEM containing 10% fetal bovine serum, 2 mM glutamine, and gentamicin (50 μg/ml) and used on the 4th to 7th day after culture. Before any treatment, confluent monolayers were switched to non-serum DMEM media containing 0.5% bovine serum albumin (BSA) for 30 min and then treated as indicated. Inhibitors were added 30 min before agonists. The cells were treated with Glu analogues added to culture medium for the indicated time periods; after that, in the case of transfected cells, the medium was replaced with DMEM/0.5% albumin.

2.3. SDS–PAGE and Western blots

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 phenylmethylsufonyl 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 proteins (100 μg as determined by the Bradford method) were resolved through a 6% 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 the particular primary antibodies indicated in each figure, followed by secondary antibodies. Immunoreactive polypeptides were detected by chemiluminescence and exposed to X-ray films. Densitometry analyses were performed and data analyzed with Prism GraphPad Software (San Diego, CA, USA).

2.4. $^{45}Ca^{2+}$ Influx

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. The Glu or D-Asp-induced influx of $^{45}Ca^{2+}$ ions was initiated at $t = 0$ by the addition of 0.5 ml solution A containing 1.5 μCi/ml solution A, Glu or Asp at the specified con-

centration. When inhibitors or modulators were tested, they were added 30 min prior to the beginning of the $^{45}Ca^{2+}$ influx assay. The reaction was stopped by aspirating the radioactive medium and washing each well within 15 s with 0.5 ml aliquots of an ice-cold solution A. The cells in the wells were then exposed for 2 h at 37 °C to 0.5 ml NaOH and an aliquot of that solution counted in a Beckmann 7800LS scintillation counter in the presence of a scintillation cocktail. Experiments were carried out at least three times in quadruplicates.

2.5. Electrophoretic mobility shift assays

Nuclear extracts were prepared as described previously (Lopez-Bayghen et al., 1996). All buffers contained a protease inhibitors cocktail to prevent nuclear factor proteolysis. Protein concentration was measured by the Bradford method (Bradford, 1976). Nuclear extracts (approximately 7.5 μg) from control or agonist-treated BGC were incubated on ice with 1 μg of poly [(dI–dC)] as a non-specific competitor (GE Healthcare) and 1 μg of [^{32}P]-end labeled double stranded oligonucleotide *chAP-1* 5′*AAGCTTGATCTGA CATCAGCTT*3′ (Aguirre et al., 2000). The reaction mixtures were incubated for 20 min on ice, electrophoresed in 8% polyacrylamide gels using a low ionic strength 0.5X TBE buffer. The gels were dried and exposed to an autoradiographic film.

2.6. Transient transfections and CAT assays

The TRE–CAT plasmid contains five copies of AP-1 site cloned in front of the thymidine kinase promoter and the CAT reporter gene. Transient transfections and CAT assays were performed in 60%

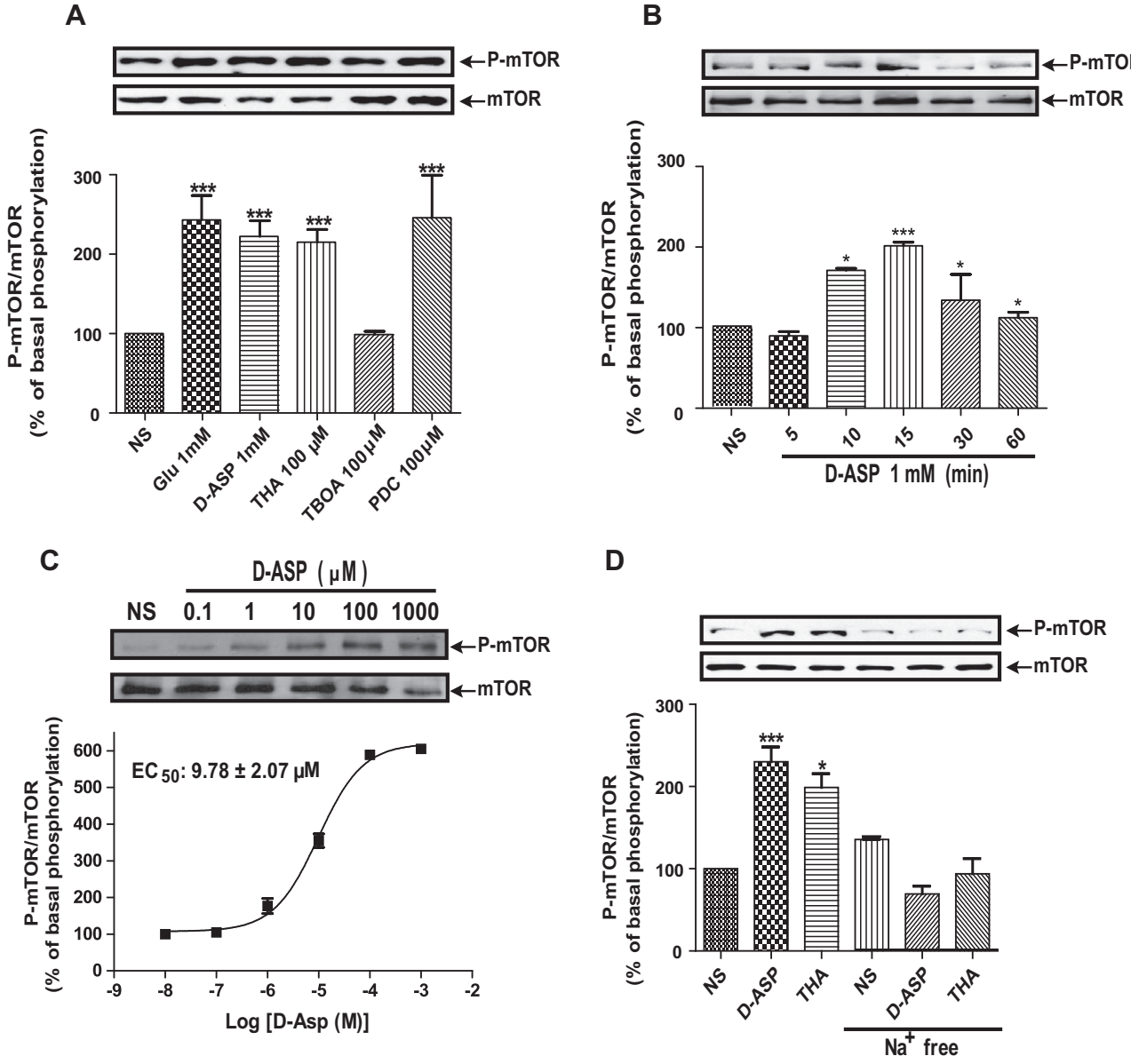

Fig. 1. D-Asp induces mTOR Ser 2448 phosphorylation in cultured Bergmann glia cells. Panel A: Confluent BGC monolayers were exposed for 15 min to the indicated concentrations of the transporter ligands and the levels of Ser 2448 phosphorylated mTOR and total mTOR were detected via Western blots as described under Section 2. Panel B: Cultured BGC were treated with 1 mM D-Asp for the indicated time periods; phospho-mTOR and total mTOR were detected as in panel A. Panel C: Cells were exposed for 15 min to increasing D-Asp concentrations. The EC_{50} was calculated after the densitometric analysis with the Prism program (GraphPad). Panel D: BGC cultures were treated for 15 min with either 1 mM D-Asp or 100 μM THA in complete or sodium-free assay buffer. Results are presented as mean values +/− standard error of at least three independent experiments. In each panel, a representative Western blot is shown. Statistical analysis was performed comparing against data from non-stimulated cells using a non-parametric one-way ANOVA (Kruskal–Wallis test) and Dunn's pos hoc test ($^{*}p < 0.05$, $^{***}p < 0.001$).

confluent BGC cultures using calcium phosphate protocol with 6 μg of p435kbpCAT reporter plasmid or 1 μg of the TRE–CAT construct. Under such conditions, the transfection efficacy was approximately 50% determined in every cell batch by an internal transfection control (β-gal). Treatment with D-Asp was performed 16 h post-transfection with indicated concentrations. Protein lysates were obtained as follows: cells were harvested in TEN buffer (40 mM Tris–HCl pH 8.0, 1 mM EDTA, 15 mM NaCl), lysed by three freeze–thaw cycles in 0.25 M Tris–HCl pH 8.0 and centrifuged at 12,000*g* for 3 min. Equal amounts of protein lysates (approximately 80 μg) were incubated with 0.25 μCi of [^{14}C]-chloramphenicol (50 mCi/mmol, GE Healthcare) and 0.8 mM Acetyl-CoA (Sigma) at 37 °C. Acetylated forms were separated by thin-layer chromatography and quantified using a Typhoon Optical Scanner (Molecular Dynamics, GE Healthcare). CAT activities were expressed as the acetylated fraction corrected for the activity in the pCAT-BASIC vector and are expressed as relative activities to non-treated control cell lysates.

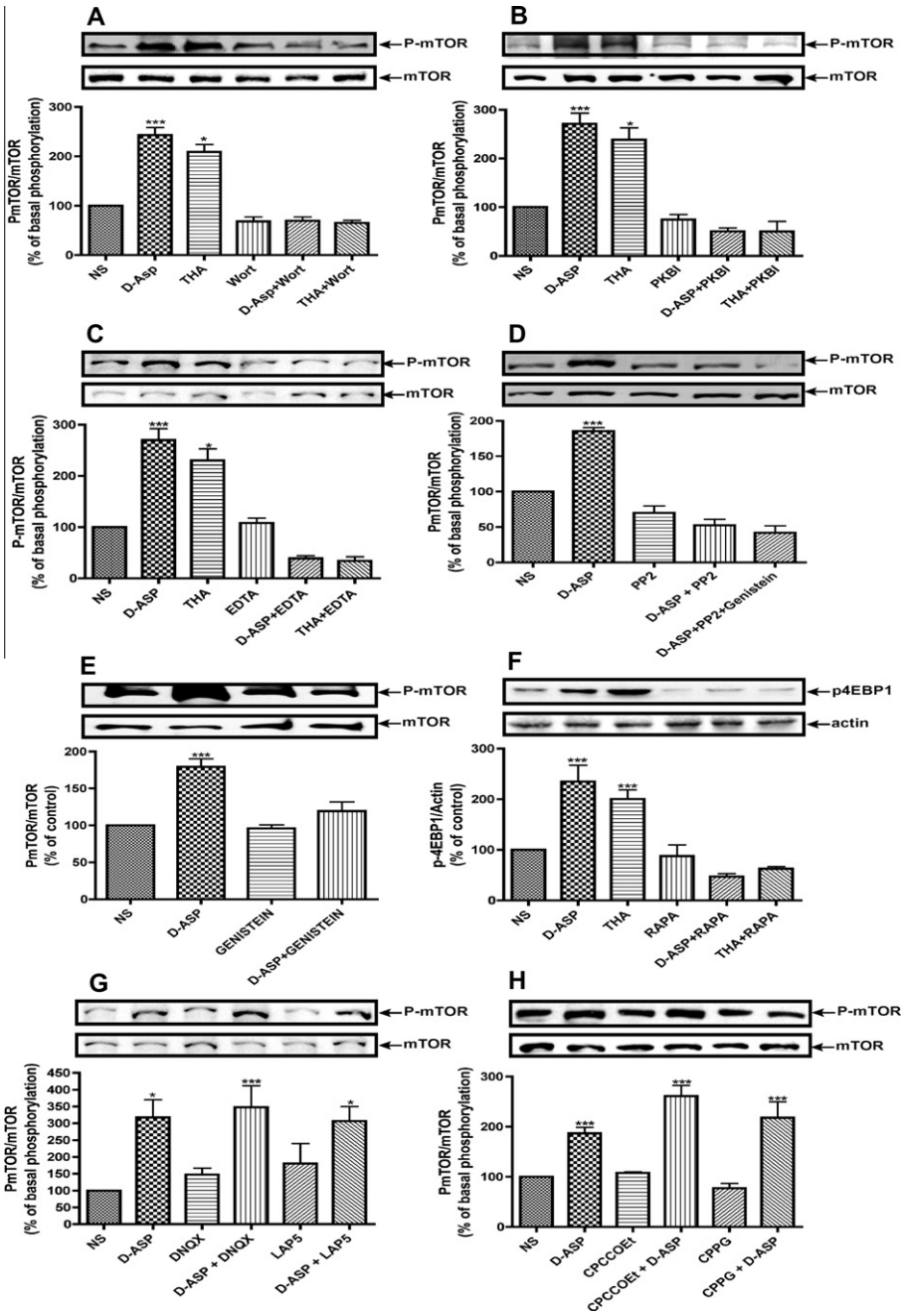

Fig. 2. D-Asp induced mTOR phosphorylation is PI3-K/Src and Ca^{2+} dependent. In all panels, Bergmann glia cultures were pre-exposed to normal medium or medium supplemented with the indicated inhibitor for 30 min and then to 1 mM D-Asp or 100 μM THA for 15 min, phosphorylated mTOR was detected as in Fig. 1. Panel A: 10 nM Wortmannin (Wort). Panel B: 100 nM PKB inhibitor IV (PKBI-IV). Panel C: Cells treated with 1 mM D-Asp or 100 μM THA in a Ca^{2+}-free medium (EDTA 500 μM). Panel D: 10 nM PP2, in the absence or presence of the tyrosine kinase inhibitor Genistein (25 μM). Panel E: 25 μM Genistein. Panel F: levels of Thr 70 phosphorylated 4EBP1 were detected after treatment with 1 mM D-Asp or 100 μM THA in the presence or absence of 100 nM Rapamicin (RAPA) for 30 min; actin was used as a loading control. Panel G: AMPA receptor antagonist DNQX (50 μM) and the NMDA receptor antagonist LAP5 (10 μM) and Panel H: metabotropic Glu receptors antagonists, for group I CPCCOEt (100 μM) and for group II/III CPPG (300 μM). All were added 30 min prior to D-Asp (1 mM, 15 min) treatment. All results are the mean values +/− the standard error of at least three independent experiments. In each case, a representative Western blot is shown. Statistical analysis was performed comparing against data obtained from non-stimulated cells using a non-parametric one-way ANOVA (Kruskal–Wallis test) and Dunn's pos hoc test ($^{*}p < 0.05$, $^{***}p < 0.001$).

2.7. Statistical analysis

Data are expressed as the mean (average) ± standard error (S.E.). A one-way analysis of variance (ANOVA) was performed to determine significant differences between conditions. When this analysis indicated significance (at the 0.05 level), post hoc Student–Newman–Keuls test analysis was used to determine which conditions were significantly different from each other with the Prism software.

3. Results

3.1. D-Aspartate induces mTOR phosphorylation in Bergmann glial cells

Translational control is the fine-tuning process of gene expression regulation. Our preliminary findings regarding Glu-dependent mTOR phosphorylation in BGC, and particularly, the fact that D-Asp treatment mimicked this effect (Zepeda et al., 2009), prompted us to characterize it. As depicted in panel A of Fig. 1, exposure of the cultured cells for 15 min to a fixed 1 mM concentration of either Glu or D-Asp, results in an increase in Ser 2448 mTOR phosphorylation. Note that this effect is reproduced by the transportable inhibitors THA and PDC but not by the non-transportable blocker TBOA, all used at a 100 μM concentration. It should also be noted that [^{3}H] D-Asp is preferred to measure Glu uptake activity in cellular systems since it is not a substrate of glutamine synthase and by these means it is not released from the cells (Gadea et al., 2004; Lau et al., 2010).

We decided to establish the time-dependence of the D-Asp effect. To this end, we exposed BGC to 1 mM D-Asp for different time periods. As clearly shown in panel B of Fig. 1, mTOR phosphorylation is maximal after a 15 min treatment. When the cells were treated with increasing D-Asp concentrations, a clear dose-dependency was found in an apparent EC_{50} of 9.78 μM (Fig. 1, panel C). Note that this value is only indicative of a transporter-mediated effect, since it does not consider the amplification inherent to any signaling cascade (Kholodenko, 2006). As Glu uptake is a Na^+-dependent process, we decided to remove Na^+ from the extracellular medium and replace it with choline chloride, and as depicted in panel D of Fig. 1, this replacement prevents D-Asp and THA effects (Fig. 1, panel D).

3.2. Signaling involved in D-Asp-mediated Ser 2448 mTOR phosphorylation and activation

To delineate the signaling cascade triggered by EAAT-1/GLAST, the sole Glu transporter expressed in cultured chick BGC (Ruiz and Ortega, 1995), we evaluated the involvement of PI-3K. This kinase is upstream of mTORC1 in several systems (Bayascas and Alessi, 2005). As expected, pretreatment of BGC monolayers with the PI-3K inhibitor wortmannin, prevented mTOR phosphorylation. (Fig. 2, panel A). Phosphorylated membrane phosphoinositides become docking sites of proteins with a pleckstrin homology domain (PH), like protein kinase B (PKB). With the use of a selective PKB inhibitor, PKB inhibitor IV (PKBI-IV), we could establish its involvement in EAAT-1/GLAST-dependent mTOR phosphorylation (Fig. 2 panel B). PI-3K activation requires its anchoring to phosphotyrosine residues and the dissociation of its catalytic subunit (p110), therefore EAAT-1/GLAST signaling necessarily leads to the activation of a tyrosine kinase (Vanhaesebroeck et al., 2010). In this context, we have previously characterized a Glu-dependent activation of the non-receptor focal adhesion kinase $pp125^{FAK}$ in this cellular system (Millan et al., 2001; Millan et al., 2004). Such activation requires Ca^{2+} influx and the activity of the non-receptor tyrosine kinase $p60^{src}$, thus we decided to explore if EAAT-1/GLAST-induced mTOR phosphorylation depends on these two phenomena. To this end, confluent BGC cultures were

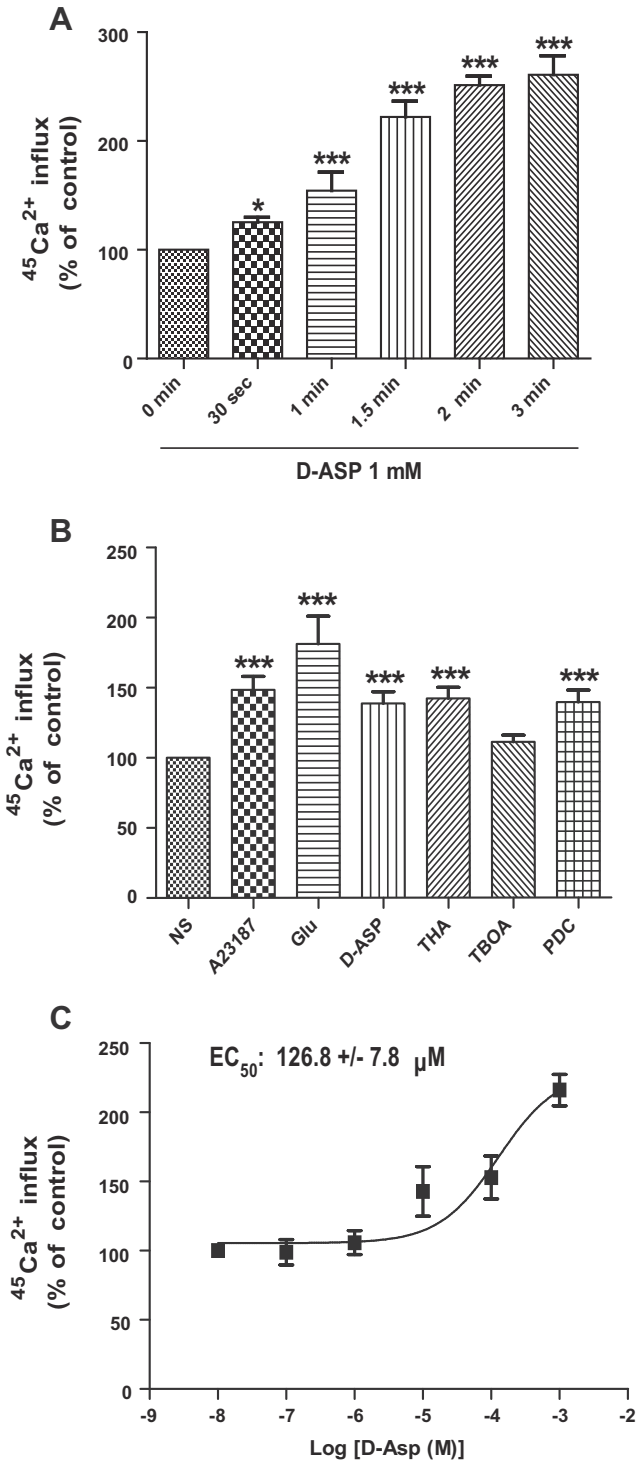

Fig. 3. D-Asp increases Ca^{2+} influx in a time and dose-dependent fashion. Panel A: Cells were incubated with 1 mM of D-Asp and the uptake performed for the indicated time periods (0, 0.5, 1, 1.5, 2 and 3 min). Panel B: $^{45}Ca^{2+}$ influx was assayed in Bergmann glia cells treated with 1 mM of Glu, 1 mM of D-Asp, 100 μM of THA, 100 μM of TBOA or 100 μM of PDC. The Ca^{2+}ionophore A23187 (10 μM) was used as a positive control. Panel C: Monolayers were incubated with increasing D-Asp concentrations (0–1 mM); in all cases, uptake time was 3 min. Graphs represent the mean values +/− standard error from at least three independent experiments in quadruplicate. Statistical analysis was performed comparing against data from non-stimulated cells using a non-parametric one-way ANOVA (Kruskal–Wallis test) and Dunn's pos hoc test (*$p < 0.05$, ***$p < 0.001$).

pre-incubated in a Ca^{2+}-free medium, supplemented with 500 μM EDTA. As shown in panel C of Fig. 2, removal of Ca^{2+} from the extracellular media prevents mTOR phosphorylation. Moreover, the $p60^{src}$ inhibitor PP2 (10 nM) prevents the D-Asp effect (Fig. 2, panel D). As expected, genistein, another tyrosine kinase inhibitor (25 μM) also prevents mTOR phosphorylation (Fig. 2, panel E). Next, in order to provide an evidence of mTORC1 activation, we examined the phosphorylation of one of its substrates, 4EBP1; the results are depicted in panel F of Fig. 2. A robust increase in Thr 70 4EBP1 phosphorylation is obtained after a 15 min exposure to D-Asp or THA. The effect is prevented by 100 nM rapamycin, demonstrating the involvement of mTORC1.

To rule out the participation of Glu receptors in the D-Asp effect, mTOR phosphorylation assays were performed in the presence of the ionotropic and metabotropic Glu receptors antagonists DNQX, LAP5, CPCCOEt and CPPG (AMPA, NMDA, group I mGluR and group II/III mGlu receptors antagonists, respectively). Neither of these antagonists prevented the D-Asp effect (Fig. 2, panels G and H), supporting the notion of an EAAT-1/GLAST mediated signaling that modulates mRNA translation.

3.3. D-Asp-induced $^{45}Ca^{2+}$ influx

The sensitivity of mTOR phosphorylation to the removal of extracellular Ca^{2+} (Fig. 2, panel C), prompted us to characterize a plausible D-Asp-induced $^{45}Ca^{2+}$ influx. Confluent BGC monolayers were exposed for different time periods to a 1 mM D-Asp solution that contained 0.4 μCi/ml of $^{45}Ca^{2+}$. As clearly shown in panel A of Fig. 3, a time-dependent $^{45}Ca^{2+}$ influx is present upon D-Asp exposure. The effect is reproduced by a Ca^{2+} ionophore, A23187 used at a 10 μM concentration, THA (100 μM) and by PDC (100 μM) another transportable inhibitor, that does not interact with Glu receptors (Amin and Pearce, 1997). Note that the non-transportable blocker TBOA fails to elicit a significant Ca^{2+} influx (Fig. 3, panel B). The dose-dependency of the Ca^{2+} influx is shown in panel C of Fig. 3, an apparent EC_{50} of 126.8 μM was obtained, indicative of a ligand–transporter interaction. In order to demonstrate that indeed, EAAT-1/GLAST triggers the Ca^{2+} influx, NaCl was replaced by choline chloride; as depicted in Fig. 4 (panel A), this is sufficient to prevent the Ca^{2+} influx. Moreover, TBOA also blocks D-Asp or THA dependent Ca^{2+} entry (Fig. 4, panel B).

Next, we decided to explore the origin of the Ca^{2+} influx. We hypothesized that the increase in intracellular Na^+ levels due to the Glu transport would lead to the activation of the Na^+/Ca^{2+} exchanger. Two blockers of the Na^+/Ca^{2+} exchanger, Amiloride (10 μM) (Knox and Ajao, 1994) and KB-R7943 (15 μM) (Elias et al., 2001) were used to challenge this possibility. The results shown in Fig. 4C indicate that amiloride reduced to non-significative levels the D-Asp induced $^{45}Ca^{2+}$ influx, whereas the more selective Na^+/Ca^{2+} exchanger blocker KB-R7943, completely abolished

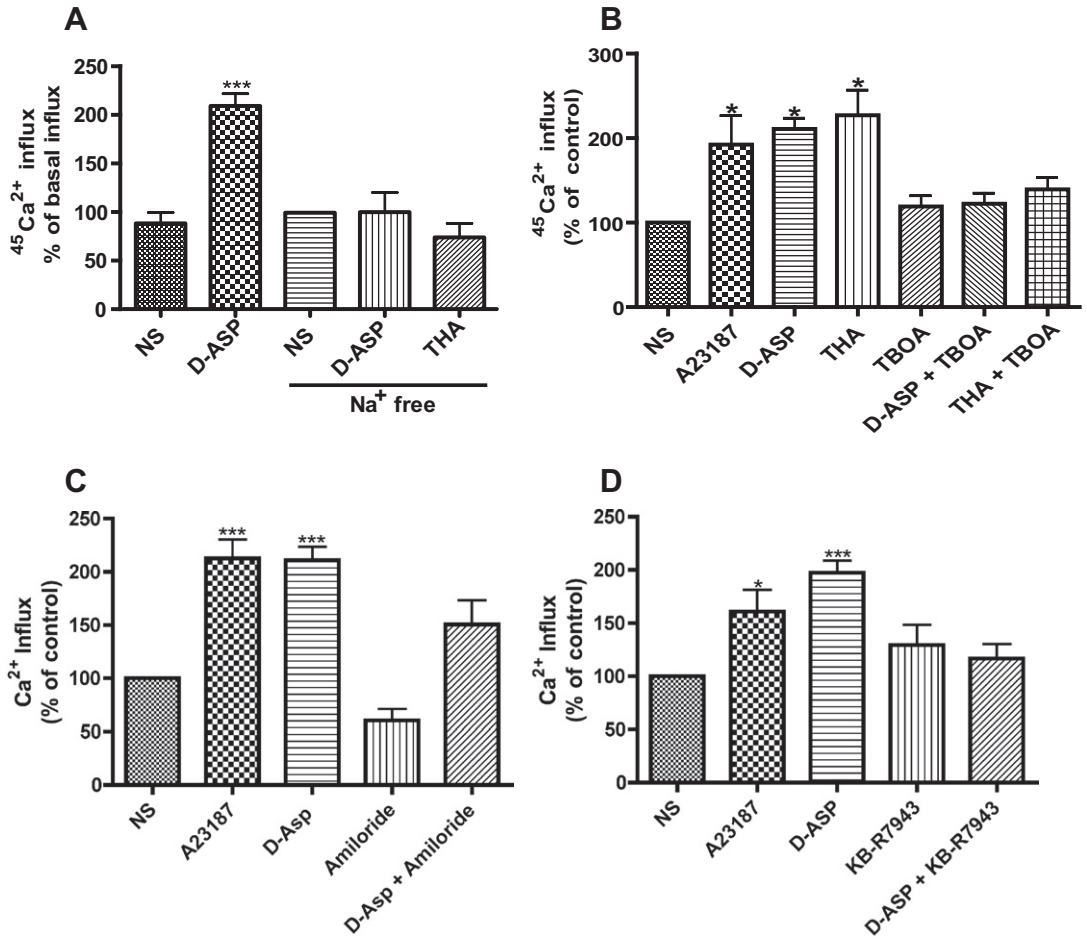

Fig. 4. D-Asp induced $^{45}Ca^{2+}$influx is EAAT-1/GLAST-dependent and involves Na^+/Ca^{2+} exchanger. Panel A: Cells were incubated with 1 mM D-Asp and 100 μM THA in regular or sodium-free assay buffer. The uptake time was 3 min. Panel B: BGC were pre-exposed for 30 min 100 μM to the non-transportable blocker, TBOA and then to 1 mM of D-Asp or 100 μM of THA. The uptake time was 3 min. Confluent BGC monolayers were pre-exposed for 30 min to the Na^+/Ca^{2+} exchanger blockers Amiloride 10 μM (Panel C) or KB-R7943 15 μM (Panel D). In all cases, $^{45}Ca^{2+}$influx was measured as in Fig. 3. Mean values +/− standard error from at least three independent experiments in triplicates. Statistical analysis: non-parametric one-way ANOVA (Kruskal–Wallis test) and Dunn's pos hoc test ($^*p < 0.05$, $^{***}p < 0.001$) against data from non-stimulated controls.

the D-Asp response (Fig. 4, panel D). Therefore one can be confident that the co-transport of Glu and Na^+ leads to the activation of the Na^+/Ca^{2+} exchanger.

3.4. D-Asp-dependent transcriptional regulation

Thus far, the results described suggest that EAAT-1/GLAST signaling is linked to translational control through mTORC1. Nevertheless, it has also been demonstrated that Glu participates in transcriptional control (Lopez-Bayghen and Ortega, 2010; Wang et al., 2007). With this in mind, we explored if the transporter is also linked to transcriptional regulation in BGC. Our first approach consisted to investigate if D-Asp or THA augmented the DNA binding activity of ubiquitous transcription factors, like Fos or Jun. Using an AP-1 sequence present in a BGC expressed gene, the chick kainate binding protein (*chkbp*) (Aguirre et al., 2000), we detected a significant enhancement of the AP-1 DNA binding activity in nuclear extracts prepared form Glu, D-Asp, THA or D-Asp + THA treated cells (Fig. 5, panel A). Note that TBOA prevents this increase and that the signal obtained after the exposure to D-Asp + THA is less prominent than the one obtained when the cells were exposed to either D-Asp or THA, suggesting that both ligands activate a common signaling pathway. The biochemical significance of the latter results, were demonstrated by gene reporter assays, BGC were transfected with an AP-1 responsive construct (TRE–CAT). A dose-dependent increase in the transcriptional activity of the reporter gene was found in the presence of D-Asp (Fig. 5, panel B). Taken together the results presented here demonstrate that the clearance of Glu from the synaptic space initiates a signal transduction cascade that presumably modifies the glial protein repertoire by regulation of the translation and transcription profiles.

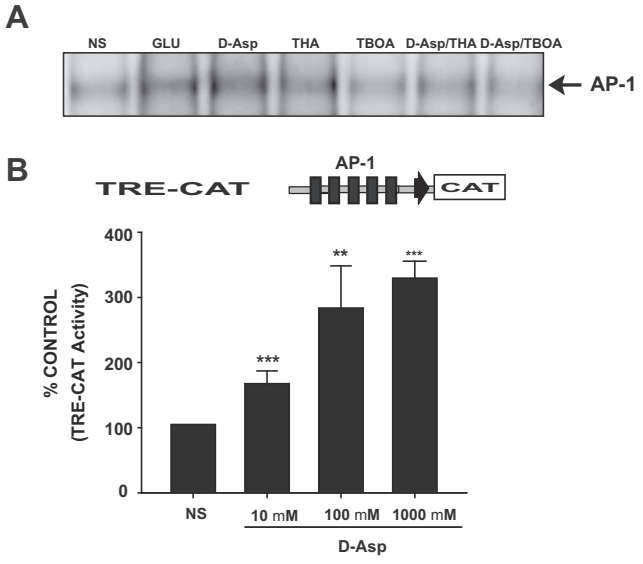

Fig. 5. D-Asp stimulation increases AP-1 DNA binding activity and up regulates an AP-1 driven construct. Panel A: Nuclear extracts were obtained from BGC treated with 1 mM Glu, 1 mM D-Asp, 100 μM THA, 100 μM TBOA, 1 mM D-Asp plus 100 μM THA or 1 mM D-Asp plus 100 μM TBOA. Gel shift assays were performed using a ^{32}P end-labeled probe (*chkbp* promoter AP-1 site, nt 337–329). A typical autoradiogram at least of three experiments is shown. Panel B: The TRE–CAT construct was transfected in BGC monolayers; 16 h post-transfection cells were exposed to the indicated D-Asp concentrations for 30 min. The transcriptional activity was determined as described under Section 2. Results are the mean value +/− the standard error of the mean of at least six independent experiments. Statistical analysis was performed comparing against non-stimulated cell using a non-parametric one-way ANOVA (Kruskal–Wallis test) and Dunn's pos hoc test ($^*p < 0.05$, $^{***}p < 0.001$).

4. Discussion

Neurotransmitter uptake is one of the most important aspects of synaptic transmission, it fact, the existence of a specific uptake system is one of the requisites that any neuroactive substance has to cover to be considered a neurotransmitter. It has been assumed that its physiological role is mainly restricted to the recycling of the neurotransmitter (Holz and Fisher, 2006). Dysfunction of the various transporter systems result in a plethora of neurological and psychiatry disorders ranging from epilepsy to depression (Kanner, 2011; Wankerl et al., 2010). Glu transporters are not the exemption and have also been described to actively participate in the homeostasis of glutamatergic transmission (Danbolt, 2001).

Glial Glu uptake and its rapid transformation into glutamine to complete the Glu/glutamine shuttle provided a biochemical framework for the involvement of these cells in glutamatergic neurotransmission (Shank and Campbell, 1984). Thereafter, the expression and characterization of glia neurotransmitter receptors paved the way into the concept of the tripartite synapse (Araque et al., 1999) and more recently the involvement of the extracellular matrix in synaptic signaling has postulated the so called tetrapartite synapse (Dityatev and Rusakov, 2011). In this context, glia cells associated to glutamatergic synapses respond to synaptic activity via receptors through the generation of Ca^{2+} waves (Muller et al., 1996) and via transporters by the Glu/glutamine shuttle and its associated inward Na^+ current (Owe et al., 2006). Of particular interest, has been glial gene expression regulation by Glu (Balazs, 2006; Gallo and Ghiani, 2000; Lopez-Bayghen and Ortega, 2010; Lopez-Bayghen et al., 2007). During our studies on the involvement of mTOR in Glu-dependent translational control in cultured BGC, we realized that part of the response was not sensitive to Glu receptors antagonists and decided to explore whether EAAT-1/GLAST, the unique Glu transporter present in these cells, was involved. A D-Asp mediated mTOR phosphorylation was found although not characterized at that time (Zepeda et al., 2009).

In an effort to fully understand these findings and to link a transporter-inward Na^+ current to a lasting response, we analyzed D-Asp-dependent translational and transcriptional regulation. We chose mTOR as an index of translational control and AP-1 DNA binding and gene reporter assays as a demonstration of transcriptional regulation. Both levels of gene expression regulation are affected by EAAT-1/GLAST in a Na^+ and Ca^{2+}-dependent manner involving PI3-K/PKB/mTOR/4EBP1/AP-1 signaling components (Aguirre et al., 2002). One could argue that neither D-Asp nor THA, TBOA or PDC are specific enough to rule out the involvement of other transporters such as GLT-1 (EAAT-2) in the described D-Asp effects. This suggestion is unlikely since BGC harbor exclusively EAAT-1/GLAST and the reported IC_{50} for dihidrokainate, a specific GLT-1 blocker is >3 mM (Ruiz and Ortega, 1995).

What could be the physiological relevance of a transporter-mediated signaling cascade in cells that express functional Glu receptors? It is difficult to have a precise answer to this question at this moment. Nevertheless, it is important to mention that exposure of mouse cerebellar slices to Glu induces inward Na^+ currents accompanied by an increase in intracellular Na^+ concentration that is marginally sensitive to CNQX but inhibited by TBOA. Furthermore, the same scenario is present upon electrical stimulation of the parallel fibers (Kirischuk et al., 2007). It is tempting to speculate that the signaling triggered by the transporters, having slower inactivation kinetics (Bazille et al., 2005) are linked to sustained biochemical responses involved in the expression of polypeptides that participate in neuron/glia coupling, such as the Na^+/K^+ ATPase that allows glia cells to clear efficiently the synaptic space. In support of this idea, is the fact that EAAT-1/GLAST associates with this plasma membrane ATPase and even, operates as an unit (Rose

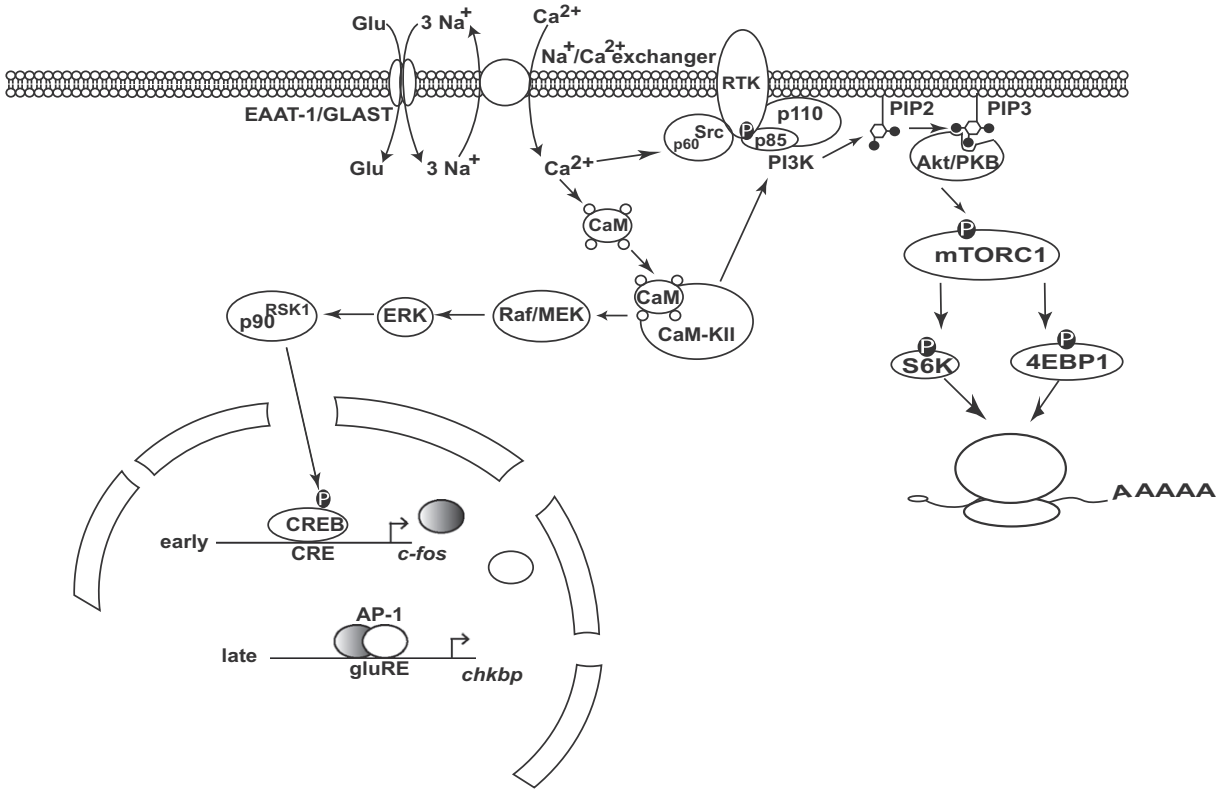

Fig. 6. Current model for EAAT-1/GLAST signaling in cultured Bergmann glia cells. Glu clearance leads to Na^+ influx, which activates the Na^+/Ca^{2+} exchanger resulting in an increase in intracellular Ca^{2+} levels, leading to the activation of a tyrosine kinase receptor (RTK) and resulting in the docking of PI3-K and $p60^{src}$. Phosphorylated phosphatidyl inositol (PIP2) recruits pleckstrin-motif containing proteins like PKB that phosphorylates mTOR, with the eventual $p70^{S6K}$ (Gonzalez-Mejia et al., 2006) and 4EBP-1 phosphorylation increasing protein synthesis. Alternatively, increased Ca^{2+} intracellular levels augments AP-1 DNA binding and this leads to the transcriptional activity of a TRE–CAT, most possibly through a Ca^{2+}/calmodulin (CaM)/Extracellular regulated kinase (ERK)/$p90^{RSK}$/CREB cascade (Aguirre et al., 2002).

et al., 2009). Other glia proteins, which expression might be regulated through the activity of the transporters are those that participate either in the Glu/glutamine shuttle like glutamine synthetase (GS) and the sodium-coupled neutral amino acid transporters (SNAT) or in the astrocyte/neuron lactate shuttle like the monocarboxylate transporters (Pellerin et al., 2007). Work currently in progress in our lab is aimed in this direction.

In conclusion, Glu transactions in glia cells are mediated through Glu receptors and transporters. From a biochemical perspective, the specificity of the cascades has started to be elucidated. A model of our present findings is depicted in Fig. 6.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neuint.2011.07.015.

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