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Novel Insights on the Regulation of the EAAT3/EAAC1 Glutamate Transporter: Interactions with the Actin Cytoskeleton and Induction by Retinoic Acid

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INDEX

Summary

In this thesis work, novel regulatory features of the glutamate transporter EAAC1, the murine counterpart of the human carrier EAAT3, have been studied. In particular, it has been demonstrated that in rat glioma C6 cells the transport activity of EAAC1 is dependent from the integrity of actin cytoskeleton. Both Latrunculin A and Cytochalasin D, two actin cytoskeleton disrupting agents, lowered the transport activity of EAAC1 decreasing its membrane abundance. However, the stimulation of EAAC1 transport activity observed after PKC-activation, was still detectable after the disruption of actin filaments. In the same study, I also observed that EAAC1 membrane trafficking was sensitive to the disorganization of microtubules, since Colchicine inhibited the aspartate influx mediated by the carrier. Altogether, these results indicated that EAAC1 interacts with both actin microfilaments and microtubules and suggested the existence of several EAAC1 intracellular pools.

Recently, several EAAC1 molecular partners have been identified that regulate either transporter trafficking or its transport activity. We have, therefore, hypothesized that also cytoskeletal components may interact with the transporter. Our attention focussed on an actin binding protein called adducin, previously found associated to other carriers and involved in their trafficking. Consistently with this hypothesis, confocal images showed areas of colocalization between an intracellular EAAC1 pool, localized in the perinuclear region, and α-adducin either before or after PKC-activation. Immunocytochemical evidence was supported by co-immunoprecipitation experiments that demonstrated the existence of a PKC-independent interaction between a pool of EAAC1 carriers and adducin. The interaction between EAAC1 and adducin was also confirmed in lysates of C6 cells transfected with α-adducin linked to a hemagglutinin (HA) epitope and immunoprecipitated with an anti-HA antibody.

To investigate more in depth the interaction between EAAC1 and adducin, we have attempted to identify experimental conditions able to increase the expression of the transporter. We found that all-trans retinoic acid (ATRA) induced a substantial overexpression of Slc1a1 and EAAC1. Moreover, in lysates of ATRA-treated cells we observed that the amount of adducin co-immunoprcipitated with EAAC1 was strongly augmented compared with lysates of untreated cells. These data confirm that adducin is a partner of the EAAC1 transporter.

The EAAC1 overexpression induced by ATRA was per se a biologically significant finding and we decided to characterize the phenomenon . The results obtained indicated that the chronic treatment with ATRA caused a four-fold increase of transport activity for anionic amino acids in C6 cells due to the overexpression of the EAAC1 protein. A proportional increase in Slc1a1 mRNA was observed with RT-qPCR while immunofluorescence showed a huge change in cell morphology

after ATRA treatement suggesting that Slc1a1 induction may be a step in a differentiation process. This possibility was supported by the induction of Plp, a typical oligodendrocytic marker, under the same conditions in which Slc1a1 expression is stimulated, while Gfap and Tubulin beta 3, two markers of, respectively, astroglial and neurones, were down regulated.

Thus, we documented for the first time the induction of EAAC1 with a differentiating agent and proposed that the expression of the transporter may represent a marker of oligodendrocytic differentiation pathway. Recent developments of this study have been aimed to confirm ATRA effects in ex-vivo models consisting of oligodendrocytes and oligodendrocytic precursor cells.

The molecular mechanisms underlying ATRA effects have been also investigated in C6 cells. The stimulation of EAAC1 transport activity after ATRA treatment was found dependent on the Retinoic Acid Receptors (RAR), since a specific RAR agonist fully mimicked ATRA effect while a specific agonist of Retinoic X Receptors (RXR) did not stimulate EAAC1 transport activity when used alone. Moreover, a RAR-specific, but not a RXR-specific, inhibitor suppressed ATRA-dependent stimulation. We also observed that ATRA effect required the synthesis of a protein intermediate and did not involve changes of the Slc1a1 mRNA half-life. Since it is known that ATRA treatment causes RARβ induction, we hypothesized that the stimulation of Slc1a1 expression requires the ATRA-mediated expression of the receptor. Indeed, we found that ATRA caused an early RARβ induction at either mRNA and protein level. More importantly, RARβ(1-2) silencing strongly inhibited Slc1a1 as well as RARβ induction by ATRA. These results point to Slc1a1 as a RARβ target gene.

Introduction

Glutamate is the most important excitatory amino acid (EAAs) in the mammalian central nervous system (CNS) and glutamatergic neurons represent essential components of many synaptic networks. Glutamate plays a crucial role not only in the support of synaptic transmission but also in the modulation of neuronal plasticity. Indeed, the synaptic compartment is endowed with several biological properties of particular relevance for CNS development and function. Among these, both long-term potentiation (LTP) and depression (LDP) are two peculiar phenomena of pivotal importance for learning and memory in which glutamatergic signaling has a crucial role (Bortolotto et al., 1999). Given the involvement of the amino acid in all these processes, which are essential for CNS development and function, it is quite obvious that its homeostasis must be finely regulated.

Glutamatergic transmission is due to the presence of several glutamate receptors subtypes located mainly in the post-synaptic region. They include the ionotropic and metabotropic receptor families. While ionotropic receptors (NMDA, AMPA and Kainate) allow the influx of ions (Ca^{2+}, Na^{+}, Cl^{-} and K^{+}) into the post-synaptic neuron, metabotropic receptors (mGluR) function via chemical second messengers to spread synaptic transmission (Figure 1).

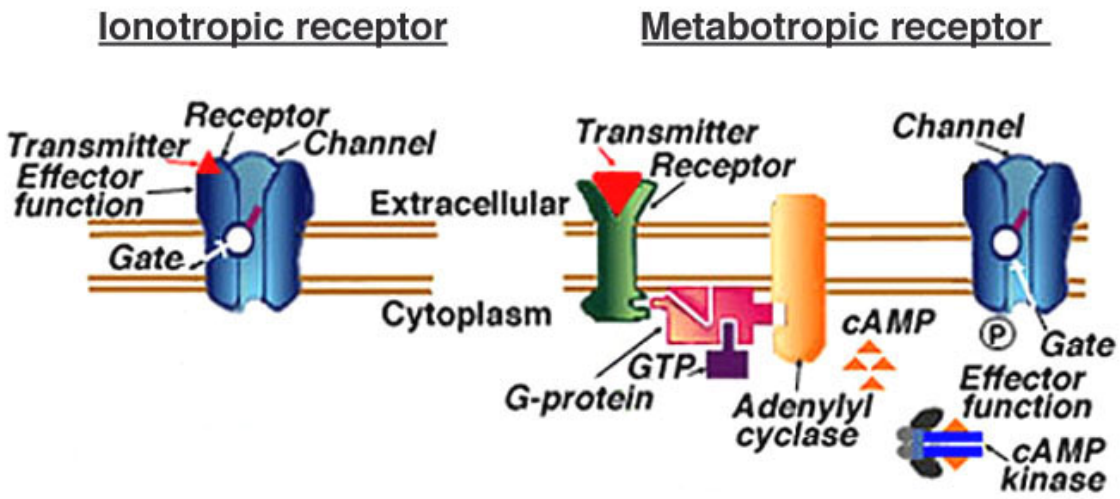

Figure 1: *Schematic representation of the Glutamate receptor (figure adapted from Kandel, E.R. et al. (1991) "Principles of neuroscience", 3rd ed. Elsevier Publishing Co, New York).*

A common feature of these receptors is the high desensitization rate, which can expose synapses to the risk of dysfunction in the case of overstimulation (Nieoullon et al., 2006). The restoration of synaptic transmission will be prevented in every condition associated to a long-lasting extracellular accumulation of glutamate, leading to the block of glutamatergic signalling.

Moreover, glutamate removal is not only important to prevent the failure of nervous signaling but also to avoid neuronal cells loss. In fact, several evidences have been reported for a long time indicating that an inpaired glutamate clearance is involved in the acute brain damage occurring after

ischemic and traumatic events (Choi, 1996; Luhmann, 1996; Han et al., 2008). Increasing concentrations of the amino acid in the brain interstitium is deleterious for the neurons inducing "excitotoxicity", via glutamate receptor overstimulation. This pathological process is mediated by a massive receptor-dependent accumulation of Ca^{2+} that triggers apoptotic cell programs or neuronal necrosis.

Along with the acute effects on cell survival, the impairment of the glutamate homeostasis seems to contribute also to the pathogenesis of several neurological disorders leading to some forms of epilepsy, as well as to psychiatric diseases such as the obsessive-compulsive disorder and schizophrenia. Moreover, numerous contributions emphasize the association between excitotoxicity and several nerodegenerative diseases such as Alzheimer and Parkinson diseases, although direct evidences of glutamate accumulation in these conditions have not yet been found. Some other studies relate also the pathogenesis of amyotrophic lateral sclerosis (ALS) with glutamate excitotoxicity. For example, it has been observed that the increase in the glutamate concentration of the cerebrospinal fluid (CSF) of ALS patients could lead to the loss of motor neurons through the activation of glutamate receptors (Sen et al., 2005). These results are consistent with previous observations indicating that the administration of anti-glutamatergic compounds can delay motor neuron injury observed in ALS (Bensimon et al., 1994; Raman et al., 2004).

In summary, glutamate clearance is a key process for the maintenance of physiological functions in the CNS and an increasing number of studies in the neuroscience field are aimed to investigate the mechanisms involved in the process, that rely exclusively on the membrane transport of the anionic amino acid. Indeed, while others neurotransmission systems are endowed with specific and efficient extracellular catabolic machineries that regulate the amount of neurotransmitter in the synaptic milieu, extracellular glutamate can be removed only through a group of Excitatory Amino Acid Transporters (EAATs). Their activity has been previously referred to the so called transport system X^-_{AG} for anionic amino acids, mainly expressed in the CNS but also found in many other tissues (Gazzola et al., 1981; Dall'Asta et al., 1983; Danbolt, 2001; Hinoi et al., 2004). To date, five isoforms of human EAATs are known (EAAT1-5), which are encoded by the Solute Carrier 1 (SLC1) gene family. Each of these transporters mediates the Na^+-dependent, high affinity uptake of aspartate and glutamate across the plasma membrane. In particular glutamate transport requires the co-transport of three Na^+ and one H^+, and the counter transport of one K^+ so as to maintain a very high concentration gradient across the plasma membrane (Gonzalez and Robinson, 2004)

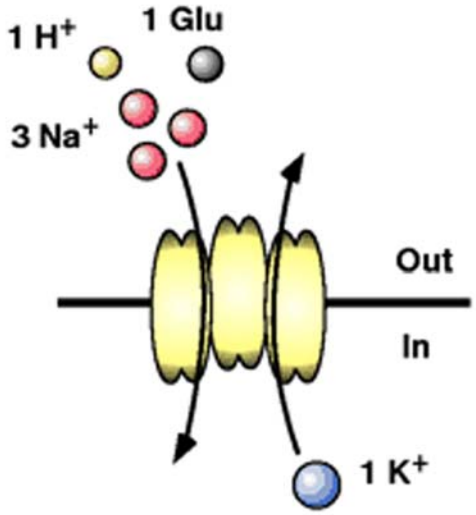

Figure 2*: Eltrogenic influx of the Glutamate within the cell is coupled by a co-transport of three Na^+ and one H^+, and the counter transport of one K (figure adapted from Gonzalez and Robinson, 2004).*

In the brain, EAAT1/GLAST, EAAT2/GLT-1 and EAAT3/EAAC1 are the most studied isoforms as they account for most of glutamate removal from the brain interstitium. While EAAT1/GLAST and EAAT2/GLT-1 are the specific glutamate transporters of astroglial cells, EAAT3/EAAC1 is a typical neuronal isoform even if it is expressed in most non neural cells.

From a functional point of view, every isoform shares the same natural anionic transport substrates (aspartate and glutamate) with apparent K_m values ranging from 5 to 20 μM. However, the EAAT isoforms responsible of the majority of glutamate uptake in the CNS are considered to be EAAT1/GLAST and EAAT2/GLT-1. In transgenic mice knocked out for these two isoforms, a strong extracellular glutamate accumulation has been reported, with neurodegenerative characteristics typical of excitotoxicity and a progressive functional impairment. Moreover, altered function of both GLT-1 and GLAST has been observed in synaptosomal fractions from a transgenic rat model of ALS accompanied by a reduced capacity for spinal cord clearance of extracellular glutamate (Dunlop et al., 2003). Therefore, glial cell populations has become the main target of several studies on the implication of excitotoxicity in neurological diseases.

However, some works provide evidences indicating that EAAT3/EAAC1 function changes could be also correlated with the onset of several neurological disease. Changes in EAAC1/EAAT3 have been reported to relate with experimental models of epilepsy (Crino et al., 2002; Proper et al., 2002; Sepkuty et al., 2002) In knockout mice for EAAT3/EAAC1, for example, although the loss of the neuronal glutamate transporter expression had no dramatic effects on neuronal survival, sporadic epileptic syndromes were detectable along with anionic aminoaciduria (Rothstein et al., 1996). More recently, studies carried out on human neocortical tissue from epileptic focal regions are consistent with previously published data showing a decrease of SLC1A1 gene expression related with a decrease in EAAT3 protein levels (Rakhade and Loeb, 2008). Such observations, however,

are not of simple interpretation given that in these epileptic forms EAAT3/EAAC1 activity seems actually abnormally increased rather than decreased. (Crino et al., 2002; Furuta et al., 2003) Alterations of the EAAT3/EAAC1 activity have been also observed in other pathological conditions. In particular, anoxia following an ischemic insult caused a significant increase in EAAT3/EAAC1 expression *in vivo* (Gottlieb et al., 2000). The increase of EAAT3/EAAC1 abundance in rat CA1 pyramidal neuron was already detectable after 8 hours from a transient brain ischemia reaching maximal levels after 1 day of reperfusion. Interestingly, induced expression of EAAT3/EAC1 was also detectable in oligodendroglial progenitor cells in subcortical white matter after both 3 and 28 days of reperfusion. However, in later postischemic periods the transporter expression levels were reduced. These results suggest the involvement of EAAT3/EAAC1 in an adaptive response aimed to prevent glutamate spill over and the subsequent brain damage following hypoxic insult. EAAT3/EAAC1 alterations seem to be also related with neurodegeneration (e.g. Alzheimer Disease), although their contribution to the pathogenesis of this condition needs other investigations (Plaitakis and Shashidharan, 2000; Meador-Woodruff et al., 2003; Molinuevo et al., 2005) Interestingly, it has been observed that Presenilin 1 affects the expression and plasma membrane activity of EAAT3/EAAC1 transporter (Yang et al., 2004).

At the light of these observations, it seems clear that further information on the cell biology and regulation of transport activity of the EAAT3/EAAC1 transporter would be highly valuable to understand both the pathogenesis of important neurological dysfunctions and the physiological responses. Thus far, many studies regarding both the cell trafficking and membrane targeting of EAAT3/EAAC1 have been performed but relatively few contributes concern regulatory mechanisms acting at the gene level. In particular, it has been observed that EAAT3/EAAC1, at variance with other EAATs, is mostly intracellular and that it could to undergo a rapid membrane redistribution triggered by several signaling cascades *in vitro* (Davis et al., 1998; Gonzalez et al., 2002; Fournier et al., 2004; Krizman-Genda et al., 2005; Gonzalez et al., 2007).

From these contributions, many of which carried out in the C6 glioma cell line, evidences have been obtained that suggest the existence of several EAAT3/EAAC1 intracellular pools. First of all it has been observed that protein kinase C (PKC) activation by phorbol esters caused an increase of the EAAT3/EAAC1 membrane abundance associated with the formation of PKCα-EAAC1 complexes. (Gonzalez et al., 2003). Subsequently, the same group (Fournier et al, 2004) showed that the increase of EAAT3/EAAC1 membrane abundance was also promoted by PDGF. However, while PKC activation blocks EAAT3/EAAC1 internalization, thus affecting the endocytic pathway and leading to the prolongation of transporter membrane shelf-life, the PDGF dependent increase in transporter abundance is mediated by an increased trafficking of a distinct EAAT3/EAAC1 pool to

the plasma membrane. Other interesting observations point to the modulatory effects of glial factors on EAAT3/EAAC1 trafficking suggesting a cross talk between glia and neurons aimed to the fine tuning of transporter activity (Lortet et al., 2008). Further studies revealed the existence of specific amino acid sequences in EAAT3/EAAC1, mainly located at the carboxyl-terminal of the protein, which are required for the PDGF-mediated membrane targeting of the transporter (Sheldon et al., 2006), suggesting that this transporter domain interacts with other proteins involved in the transporter trafficking.

Indeed, as other EAAT carriers, also EAAT3/EAAC1 transporter is known to interact with other proteins. One of these molecular partners is GTRAP3-18 (Lin et al., 2001; Ruggiero et al., 2008) while others are members of the SNARE proteins (Fournier and Robinson, 2006; Yu et al., 2006). These proteins have been involved in the sorting of the carrier proteins and in the modulation of transport activity through changes in the apparent affinity (K_m) of the transporter. Interestingly, most of these proteins have been related to cytoskeletal components, raising the possibility that one or more pools of EAAT3/EAAC1 carriers interact with cell cytoskeleton. This possibility has been investigated in the first contribution produced during this thesis work.

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PKC-dependent stimulation of EAAT3 glutamate transporter does not require the integrity of actin cytoskeleton

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Abstract

The activity and the membrane expression of EAAT3 glutamate transporter are stimulated upon PKC activation by phorbol esters in C6 rat glioma cells. To investigate the role of cytoskeleton in these effects, we have employed actin-perturbing toxins and found that the perturbation of actin cytoskeleton inhibits basal but not phorbol-stimulated EAAT3 activity and membrane trafficking.

In the absence of phorbols, latrunculin A, a toxin that disassembles actin cytoskeleton, produced a rapid inhibition of EAAT3 activity, due to a decrease in transport V_{max}. The inhibitory effect was fully reversible and was not detected for other sodium dependent transport systems for amino acids. However, latrunculin did not prevent the increase in transport caused by phorbol esters and, moreover, cells pretreated with phorbols were resistant to the inhibitory effect of the toxin on EAAT3 activity. Biotinylation experiments indicated that the inhibitory effect of latrunculin was attributable to a decreased expression of the carrier on the membrane, while the toxin did not suppress the PKC-dependent increase in EAAT3 membrane abundance. Latrunculin A effects on EAAT3 were shared by cytochalasin D, a toxin that disorganizes actin filaments with a distinct mechanism of action. On the contrary, a small, but significant, increase of EAAT3 activity was observed upon incubation with jasplakinolide, a drug that stabilizes actin microfilaments. Also jasplakinolide, however, did not hinder phorbol-dependent stimulation of aspartate transport. Colchicine, a toxin that disrupts microtubules, also lowered EAAT3 activity without preventing transport stimulation by phorbols, while microtubule stabilization by paclitaxel led to an increase in aspartate transport.

It is concluded that, in C6 cells, the PKC-mediated stimulatory effects on EAAT3 are cytoskeleton-independent while, in the absence of phorbols, the transporter is partially inhibited by the disorganization of either actin microfilaments or microtubules. These results suggest that EAAT3 trafficking in C6 cells involves different pools of transporters.

Keywords: Aspartate; Cytochalasin D; Jasplakinolide; Latrunculin A; Microtubules; Phorbols; Trafficking

1. Introduction

Although derived from a glial tumor, rat C6 cells transport glutamate mainly through the neuronal type transporter EAAT3/EAAC1. While this transporter is expressed at high levels in these cells, the typical glial transporters EAAT1/GLAST or EAAT2/GLT1 are barely present (Dowd et al., 1996; Palos et al., 1996). As a consequence, C6 cells have become a privileged model for studying the cell biology of EAAT3 and have been used to characterize several regulatory mechanisms affecting the expression and/or the activity of the transporter. Among these regulations, the increase of EAAT3 activity upon exposure to phorbol esters has been analysed in depth (Casado et al., 1993; Dowd and Robinson, 1996; Davis et al., 1998; Gonzalez et al., 2003; Fournier et al., 2004). As originally described in cultured human fibroblasts (Franchi-Gazzola et al., 1990), phorbol-induced transport stimulation in C6 cells is rapid, protein synthesis-independent, and prevented by inhibitors of classical or novel isoforms of protein kinase C (PKC). The precise mechanism of the increase is still debated and, possibly, heterogeneous. Indeed, some reports suggest a direct interaction between the transporter and PKCα that would lead to phosphorylation of EAAT3 (Casado et al., 1993; Gonzalez et al., 2003) and to its redistribution to the cell surface (Casado et al., 1993; Gonzalez et al., 2003). Another contribution (Gonzalez et al., 2002) describes the increase in EAAT3 intrinsic transport activity dependent on the PKCε isozyme, previously implied in the stimulation of anionic amino acid transport also in non nervous cells (Franchi-Gazzola et al., 1996).

Recently, the existence of different pools of EAAT3 transporters has been invoked to explain these discrepancies (Fournier et al., 2004). According to this view, a pool of EAAT3 proteins would be redistributed to the cell surface following PKC activation, whereas another, PKC-insensitive fraction of transporters would be stimulated by PDGF (Sims et al., 2000). Moreover, phorbols, but not PDGF, block EAAT3 internalisation,

suggesting that PKC activation stimulates glutamate transport hindering carrier endocytosis (Fournier et al., 2004).

The role of cytoskeleton in these regulatory phenomena has not been characterized yet although interactions with cytoskeletal components have been described for other EAAT transporters, such as EAAT1 (Duan et al., 1999). Two recent contributions suggest that also some regulatory features of EAAT3 activity may be dependent on cytoskeleton (Najimi et al., 2002; Najimi et al., 2005). Those results raise the possibility that the actin cytoskeleton is involved in PKC-mediated changes in EAAT3 activity or trafficking. To evaluate this hypothesis, we have examined the effects of cytoskeletal perturbing agents on EAAT3 activity under basal and phorbol-stimulated conditions and demonstrate here that the disorganization of actin cytoskeleton inhibits basal, but not phorbol-stimulated, EAAT3 activity and membrane expression.

2. Experimental procedures

2.1 Cells

The rat central nervous system-derived cell line C6 glioma cells was obtained from American Type Culture Collection (Rockville, MD). Cells were grown at 37°C and 5% CO_2 in Dulbecco's modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 4 mM glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin. Cells were passaged less than 10 times with no apparent change in morphology or sensitivity to treatments.

2.2 Experimental treatments

Drugs were obtained from Sigma-Aldrich, Milan, Italy, unless otherwise specified. Final and stock concentrations were 0.6 μM and 300 μM (in DMSO) for latrunculin A; 5 μM and 2.5 mM (in DMSO) for cytochalasin D; 1 μM and1 mM (in DMSO) for colchicine; 1 μM and 1 mM for paclitaxel (in DMSO); 150 nM and 1 mM (in ethanol) for phorbol 12,13-dibutyrate (PDBu). Jasplakinolide (obtained from Inalco SpA, Milan), was diluted in DMSO at 500 μM and employed at the final concentrations detailed in Figure 7. Vehicles were added to control cells at the maximal concentration employed for drug treatments.

2.3 Visualization of actin in cultured cells

The actin cytoskeleton was visualized by staining the cells with AlexaFluor-phalloidin (Molecular Probes, Eugene, OR). C6 cells were seeded on coverslips at a density of $5 \cdot 10^3$ cells/cm^2 and treated after 24h. After the experimental treatments, cells were rinsed in PBS and fixed for 10 min in 3.7% paraformaldehyde in PBS. After two further rinses, cells were permeabilized with a 10-min incubation in 0.1% Triton X-100 in PBS. Cells were then incubated for 20 min at 37°C with AlexaFluor-phalloidin (15 U/ml) in PBS and washed three times in PBS. The last wash was supplemented with propidium iodide (4μg/ml) to

stain the nuclei. The morphology of treated cells was then studied with a confocal system (Multiprobe 2001, Molecular Dynamics, Sunnydale, CA) employing a 60x objective. Phalloidin signal was acquired at a λ_{ex} of 488 nm and the emission recorded through a 510 nm primary beamsplitter and a 530 nm dichroic filter. Propidium iodide signal was recorded from the emission over 570 nm selected employing a epifluorescence long pass filter.

2.4 Determination of transport activity

The determination of EAAT3 transport activity was performed through the measurement of the initial influx of L-aspartate, a high affinity, specific substrate of EAAT transporters (Gazzola et al., 1981; Dall'Asta et al., 1983). For the determination of aspartate influx, C6 glioma cells were seeded into Corning 96-well multidish plates (Celbio, Milan, Italy) at a density of 10-15.000 cells/well. After 2d, experimental treatment was performed, as detailed for each experiment. At the end of the treatment, cells were incubated in Earle's Balanced Salt Solution (EBSS), buffered at 7.4 with 20 mM HEPES/NaOH and supplemented with [^{3}H]L-aspartate (1 μM, 1 μCi/ml) for 1 min, unless otherwise stated. Preliminary experiments had ascertained that, under the conditions adopted, aspartate transport was linear up to 90 s (not shown). At the end of the assay, multiwell dishes were rapidly washed twice (< 10 s) with ice-cold 300 mM urea and cell monolayers were extracted with 50 μl of absolute ethanol. The extracts were then added to 200 μl of scintillation fluid and counted for radioactivity with a Wallac Trilux liquid scintillation spectrometer (Perkin-Elmer, Boston, MA). Cell proteins were determined with a modified Lowry procedure directly in the well and measured with a Wallac Victor^2 Multilabel Counter (Perkin-Elmer).

For the determination of kinetic constants, the uptake of increasing concentrations of L-Asp, from 0.4 μM to 200 μM, was determined. After subtraction of the diffusive component,

assessed from the transport of 2 mM L-Asp, data were fitted with a non linear regression analysis to the equation

$$v = \frac{V_{max} * [S]}{K_m + [S]} \quad \text{(Eq. 1)}$$

that describes L-Asp transport as the result of a single component. The analysis was performed with a GraphPad Prism3™ software.

The uptake of L-proline, as an indicator of the activity of SNAT transporters (Mackenzie and Erickson, 2004), and of L-threonine or L-glutamine, as indicators of ASCT activity (Kanai and Hediger, 2004) , were determined in cells grown in 96-well microplates under conditions detailed in the legend of Figure 8.

2.5 Biotinylation and Western Blot

Biotinylation of cell surface proteins was performed as previously described (Davis et al., 1998) with slight modifications. Briefly, cultures of C6 cells were grown to subconfluence on 10-cm^2 tissue culture plates. After the experimental treatment, cells were rinsed twice in PBS with Ca and Mg. The plates were then incubated with 2 ml of biotin solution (EZ-Link Sulfo-NHS-LC-Biotin, Pierce, 1 mg/ml in Ca/Mg-PBS) for 30 min at 4°C with gentle shaking. The cells were then washed 3 times with ice-cold Ca/Mg-PBS containing 100 mM glycine, incubated in the same solution for 45 min at 4 °C with gentle agitation, and lysed in 1 ml of RIPA buffer (100 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Igepal CA 630, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate) containing a cocktail of protease inhibitors (Complete, Mini, EDTA-free, Roche) for 1 hr at 4 °C with vigorous

shaking. Lysates were transferred in Eppendorf tubes, sonicated for 15 sec, and centrifuged at 16000*g* for 30 min at 4 °C.

After quantification (Bio-Rad protein assay), 150 µl of each sample, containing the same amount of proteins, were mixed with 125 µl of NeutrAvidin slurry-beads and kept over-night at 4°C in gentle shaking. The mixture was centrifuged at 10000*g* for 10 min, and the supernatant was kept as non-biotinylated fraction for the Western blot after addition of 150 µl of Laemmli buffer 4x just before loading (intracellular fraction). The pellet was washed once with RIPA buffer, once with a high-salt solution containing 50 mM Tris-HCl, 500 mM NaCl, 5 mM EDTA, 0.1% Igepal CA 630, three times with a low-salt solution containing 50 mM Tris-HCl, resuspended in 150 µl of Laemmli buffer 2x (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 0.2 M 2-mercaptoethanol) for 10 min with occasional shaking to elute the biotinylated proteins, and centrifuged briefly at 12000*g*. In parallel, 150 µl of total lysate of each sample was eluted whit the same volume of Laemmli buffer 4x. Equal volumes of total lysate, intracellular fraction, and membrane samples were warmed at 65°C for 30 min and loaded on a 8% gel for SDS-PAGE. After the electrophoresis, proteins were transferred to polyvinylidene difluoride membranes (Bio-rad). Non-specific binding sites were blocked with an incubation in Tris-buffer saline (TBS; 50 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 1% casein, 0.33% gelatin, 3% bovine serum albumin for 2h at room temperature. The blots were then exposed to anti-EAAT3 polyclonal antiserum (1:200), obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), diluted in blocking solution overnight at 4°C. After washing, the blots were exposed to horseradish peroxidase-conjugated anti-rabbit IgG diluted 1:1000 in blocking solution for 1h. For standardization of the total cell lysate and the intracellular fraction, stripped membranes were exposed to polyclonal anti-rabbit antiserum against β-tubulin (Santa Cruz Biotechnology, 1:500). The membranes were washed and the immunoreactivity visualized with enhanced chemiluminescence (Amersham Pharmacia Biotech Italia, Milan, Italy).

2.6 Statistical analysis

Statistical analysis of data utilized either ANOVA or unpaired Student's *t*-tests depending on the number of groups compared. $P < 0.05$ were considered significant.

2.7 Materials

Serum and culture medium were obtained from Celbio Euroclone (Milan, Italy). L-[2,3-^{3}H]Aspartic acid (39 Ci/mmol), L-[5-^{3}H]proline (31 Ci/mmol), L-[G-3H]glutamine (54 Ci/mmol), and L-[3-^{3}H]-threonine (15 Ci/mmol) were purchased from Amersham Pharmacia Biotech Italia. Sigma was the source of the other chemicals whenever not stated otherwise.

3. Results

3.1 Effect of anti-actin toxins on microfilaments in C6 cells

At submicromolar concentrations latrunculin A, a toxin from the Red Sea sponge *Latrunculia magnifica*, complexes with monomeric actin, thus lowering the pool of free actin available for polymerization, disorganizing actin microfilaments, and producing characteristic morphological changes (Peterson and Mitchison, 2002). The effect of a 15-min treatment of C6 cells with 0.6 μM latrunculin A is shown in Fig. 1, Panel B. Compared with control, untreated cells (Panel A), the toxin caused the disappearance of most actin filaments with an apparent clumping of actin in cytoplasmic masses. Cell density, however, was substantially comparable in control and treated cultures. The effects of cytochalasin D, a fungal metabolite that disorganizes actin cytoskeleton preventing the elongation of microfilaments (Peterson and Mitchison, 2002), are shown in Panel C. In this case, cells still exhibited detectable, although severely shortened, actin microfilaments, mostly grouped in the perinuclear region. Also cytochalasin, however, did not cause marked cell loss under the conditions adopted.

3.2 Effect of latrunculin A on EAAT3 activity

Fig. 2, Panel A reports the results of an experiment in which EAAT3 activity, determined as L-Asp uptake, was measured in C6 cells incubated in the presence of 0.6 μM latrunculin for increasing times. The incubation in the presence of the cytoskeletal modifier produced a rapid decrease of EAAT3 transport activity. The decrease was already detectable at the first time point employed (3 min of latrunculin treatment) and was maximal at times longer than 10 min, with an inhibition ranging from 30% to 40% in the various experiments.

The effect of latrunculin on EAAT3 activity was fully reversible. Indeed, if medium supplemented with the toxin was replaced by latrunculin-free growth medium, aspartate

transport rapidly recovered and was fully restored after 30 min to stabilize thereafter (Fig. 2, Panel B).

The kinetic analysis of L-Asp uptake, showed in Fig. 2, Panel C, indicated that latrunculin inhibited EAAT3 activity through a decrease in transport V_{max} (from 1056 ± 29 to 514 ± 15 pmol/mg protein/min), while K_m was not affected by the drug (17.4 ± 1.38 *vs.* 16.1 ± 1.42 μM in control and latrunculin-treated cells, respectively).

3.3 Effect of latrunculin A on PKC-mediated stimulation of EAAT3 activity and surface expression

The activation of PKC by phorbol esters rapidly stimulates the activity of EAAT3 in C6 cells (Casado et al., 1993; Fournier et al., 2004). In our hands, the PKC-dependent increase in transport activity was rapid, reaching a maximum of 80-100% after 10 min of exposure to phorbol 12,13-dibutyrate (PDBu), to decrease slowly thereafter (not shown). The kinetic analysis indicated that the increase in aspartate influx depended upon an increase in transport V_{max} (from 1026 ± 32 to 1645 ± 38 pmol/mg protein/min) whereas K_m was slightly higher in cells exposed to phorbols (16.9 ± 1.79 *vs.* 19.1 ± 1.46 μM in control and treated cells, respectively).

The preincubation with latrunculin (Fig. 3) up to 60 min did not hinder the stimulation of aspartate transport triggered by PKC activation. Indeed, cells pretreated with the drug still exhibited a clear-cut stimulation of EAAT3 activity upon 10-min treatment with PDBu, although, in the absence of the phorbol, the toxin clearly inhibited aspartate transport.

Figure 3 presents the results of a Western Blot analysis of EAAT3 expression in C6 cells treated with latrunculin A and/or phorbol ester. Under the experimental conditions adopted for cell lysis and protein electrophoresis, most of EAAT3 was in multimer form. As expected (Fournier et al., 2004), PDBu caused a marked increase in the membrane expression of EAAT3 and, in parallel, a decrease of the transporter protein in the

intracellular fraction. In latrunculin-treated cells EAAT3 abundance on plasma membrane was significantly decreased (Fig. 4, Panel A) and, consistently, the abundance of the transporter in the intracellular compartment increased. However, even in latrunculin-treated cells, PDBu caused an evident increase in surface expression of EAAT3. The densitometric analysis (Fig. 4, Panel B) indicated that the relative changes in the membrane fraction of EAAT3 transporters were larger than the modifications detected in the intracellular fraction. However, the overall amount of EAAT3 transporter expressed in the total cell lysate was not appreciably modified either by PDBu or by latrunculin treatment.

The effect of PDBu pre-treatment on latrunculin-dependent inhibition of EAAT3 activity is shown in Fig. 5. The results indicate that, in cells exposed to the phorbol ester for 10 min, a 5-min treatment with latrunculin had no inhibitory effect on EAAT3 activity that, rather, appeared to be slightly stimulated by the toxin. In the same experiment, the toxin inhibited EAAT3 activity in cells not treated with PDBu. Phase-contrast microscopy indicated that, however, PDBu did not prevent the morphological changes caused by latrunculin (results not shown).

3.4 Effect of other modifiers of actin cytoskeleton

The effects of cytochalasin D on EAAT3 activity and expression are shown in Fig. 6, Panel A. As latrunculin A, also cytochalasin D produced a decrease in basal EAAT3 transport activity and did not prevent its stimulation by PDBu. As for latrunculin, also for cytochalasin D transport inhibition was associated with a decrease in V_{max} from 1101 $\pm$ 31 to 667 $\pm$ 25 pmol/mg prot/min (not shown). Cytochalasin effects on EAAT3 membrane expression (Fig. 6, Panel B) were comparable to those observed with latrunculin. Under basal conditions, the toxin lowered the surface expression of EAAT3, although it did not prevent the increase in carrier abundance observed upon treatment with PDBu.

Jasplakinolide, a cell permeant drug that is described to stabilize actin filaments (Peterson and Mitchison, 2002), had a biphasic effect on EAAT3 activity (Fig. 7). At doses up to 500 nM, jasplakinolide increased aspartate transport, while at 1 μM the toxin produced a decrease in aspartate transport. However, at any concentration tested, jasplakinolide did not prevent aspartate transport stimulation by PDBu.

3.5 Specificity of latrunculin effects on EAAT3 activity

To assess the specificity of latrunculin effects on EAAT3, the activity of other sodium-dependent transporters for amino acids were measured in C6 cells treated with the toxin for periods sufficient to detect a significant decrease of glutamate uptake. Treatment with latrunculin did not inhibit the transport of L-proline (Fig. 8, Panel A), a substrate of system A (SNAT transporters) in C6 cells (Zafra et al., 1994). Moreover, the cytoskeletal toxin did not significantly hinder the transport of either L-threonine (Panel B) or L-glutamine (Panel C), two substrates of system ASC (ASCT2 transporter) in C6 cells (Dolinska et al., 2003). PDBu had only marginal inhibitory effects on threonine and glutamine transport, while it was ineffective on proline uptake.

3.6 Effect of microtubule toxins on EAAT3 activity

The disruption of microtubules had effects on EAAT3 activity comparable to those exhibited by latrunculin A or cytochalasin D. Cells treated with colchicines, indeed, had lowered aspartate influx although, even under this condition, EAAT3 activity was still enhanced by PDBu (Fig. 9). On the contrary, the microtubule stabilizing agent paclitaxel had a modest, although significant, stimulatory effect on aspartate influx.

4. Discussion

4.1 PKC effects on EAAT3 glutamate transporter do not require cytoskeletal integrity.

The stimulation of EAAT3 activity upon PKC activation by phorbol esters is a well known phenomenon and may have profound physiological consequences (Robinson, 2002). One of the mechanisms invoked to explain the effect of phorbols is the increase in the membrane expression of the transporter (Davis et al., 1998), attributed to changes in EAAT3 endocytosis (Gonzalez et al., 2003). The present report demonstrates that, in phorbol-treated C6 glioma cells, the integrity of the actin cytoskeleton is not required for either the stimulation of EAAT3 activity or the increase of EAAT3 membrane expression caused by PKC activation. This conclusion is based on the demonstration that latrunculin A, cytochalasin D, and jasplakinolide, three toxins which profoundly affect actin organization with distinct mechanisms and, possibly, divergent effects, do not interfere with EAAT3 stimulation by phorbols. Moreover, also disruption of microtubules by colchicine does not prevent PKC-dependent stimulation of glutamate transport that appears, therefore, a cytoskeleton-independent phenomenon.

Multiple kinases affect EAAT3 activity and membrane expression (Schniepp et al., 2004; Guillet et al., 2005). However, the PKC-dependent up-regulation of the transporter trafficking towards plasma membrane is a peculiar feature of EAAT3. Indeed, the surface expression of other members of EAAT family, such as EAAT1 and EAAT2, is down regulated by phorbols (Guillet et al., 2005). Moreover, PKC activation promotes the internalization pathway of several other transporters, such as NaPi-II (Forster et al., 1999), the dopamine transporter DAT (Chang et al., 2001), the norepinephrine transporter NET (Apparsundaram et al., 1998), the dicarboxylate acids transporter NaDC-1 (Pajor and Sun, 1999), and the organic anion transporter hOAT1(Wolff et al., 2003). In some of these contributions actin perturbing toxins have been employed to assess the dependence of PKC-dependent regulations on cytoskeleton. For instance, actin-modifying toxins prevent

changes in phorbol-induced internalization of NaDC-1 (Pajor and Sun, 1999) but do not affect hOAT1 inhibition (Wolff et al., 2003).

4.2 Actin disorganization inhibits EAAT3 activity and membrane trafficking in phorbol-untreated cells.

Under basal conditions, i.e. in the absence of PKC-activation promoted by phorbol treatment, the membrane expression and the activity of EAAT3 are partially inhibited if actin cytoskeleton is disorganized. The inhibition is partial and occurs with both cytochalasin D and latrunculin A, notwithstanding the distinct mechanisms employed by the two toxins to disorganize the actin network (Peterson and Mitchison, 2002). This result is unexpected since two recent studies on the same cell model employed here observed no inhibition of basal EAAT3 activity upon cytochalasin D treatment, although implied actin cytoskeleton in the up regulation of EAAT3 by neurotensin or endothelin (Najimi et al., 2002; Najimi et al., 2005). Since those authors use cytochalasin at a concentration 6-fold higher than that used in the present report, the lack of effect of the toxin cannot be attributed to a difference in the concentrations employed but, rather, to the relatively short cytochalasin treatment time (15 min) adopted in those contributions.

At least for latrunculin, EAAT3 inhibition should not be ascribed to gross changes in membrane potential or transmembrane sodium gradient. Indeed, latrunculin A does not lower the activity of other sodium- and membrane potential-dependent amino acid transporters, such as ASCT2, for the transport of glutamine and threonine (Dolinska et al., 2003), and SNAT transporters for the transport of L-proline (Zafra et al., 1994). Moreover, latrunculin effect is not due to an irreversible damage of treated cells, given that the removal of the toxin leads to a rapid and complete restoration of transport activity (Fig. 2B). Instead, the inhibition of transport correlates with a decrease in EAAT3

immunoreactivity in surface protein pool, suggesting that actin network is involved in the trafficking of a population of EAAT3 transporters towards plasma membrane.

The interaction between EAAT3 transporters and actin cytoskeleton seems to be PKC-sensitive. The intriguing resistance of EAAT3 activity to latrunculin inhibition, exhibited by phorbol-pretreated cells (see Figure 5), suggests, indeed, that PKC-activation renders EAAT3 trafficking completely independent from the organization status of actin.

The inhibition of the basal transport activity by toxin-mediated cytoskeletal disruption has been observed for other carriers, such as hOAT1 (Wolff et al., 2003) and the serotonin transporter SET (Sakai et al., 2000). These results have been taken as an evidence for an actin-sensitive insertion step in the continuous recycling of the transporter between plasma membrane and intracellular compartments (Wolff et al., 2003). Consistently, a pool of EAAT3 transporters in thus far unidentified intracellular compartments has been recently visualized using an EAAT3-GFP construct (Otis et al., 2004) or anti-EAAT3 antibodies (Guillet et al., 2005). Changes in the intracellular distribution of EAAT transporters may underlie modifications in anionic amino acid transport observed in the absence of changes of overall expression of EAAT3, as recently described in ammonia-exposed rat cerebellar granule neurons (Chan et al., 2003).

Jasplakinolide, a toxin originally described as an actin stabilizer (Bubb et al., 1994), produces a small, but reproducible, stimulation of EAAT3 activity under either basal (i.e. in the absence of PDBu) or PDBu-stimulated conditions if employed at concentrations up to 500 nM. The peculiar mechanism of action of the toxin, which can lead to a paradoxical actin disorganization at high concentrations (Bubb et al., 2000), may underlie the decrease in EAAT3 activity detected at 1 μM jasplakinolide. Under this condition, morphological changes compatible with the disorganization of actin cytoskeleton are apparent in C6 cells (results not shown).

4.3 Conclusions.

The disruption of actin cytoskeleton of C6 cells affects EAAT3 membrane expression and activity in cells maintained under basal conditions but not in cells stimulated with phorbols. These results suggest that a portion of EAAT3 transporters are dependent on actin filaments for their trafficking to membrane and are, therefore, associated with actin cytoskeleton. Since comparable results are obtained, at least for EAAT3 activity, when microtubules are disrupted, it is possible that also microtubules are involved in EAAT3 trafficking. Although further studies will be necessary to identify which cytoskeletal proteins are linked to EAAT3 transporters, these data are consistent with the presence of different trafficking pathways and possibly, distinct pools of EAAT3 transporters in C6 cells.

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References

Apparsundaram, S., Schroeter, S., Giovanetti, E., Blakely, R. D., 1998. Acute regulation of norepinephrine transport: II. PKC-modulated surface expression of human norepinephrine transporter proteins. J. Pharmacol. Exp. Ther. 287, 744-751.

Bubb, M. R., Senderowicz, A. M., Sausville, E. A., Duncan, K. L., Korn, E. D., 1994. Jasplakinolide, a cytotoxic natural product, induces actin polymerization and competitively inhibits the binding of phalloidin to F-actin. J. Biol. Chem. 269, 14869-14871.

Bubb, M. R., Spector, I., Beyer, B. B., Fosen, K. M., 2000. Effects of jasplakinolide on the kinetics of actin polymerization. An explanation for certain in vivo observations. J. Biol. Chem. 275, 5163-5170.

Casado, M., Bendahan, A., Zafra, F., Danbolt, N. C., Aragon, C., Gimenez, C., Kanner, B. I., 1993. Phosphorylation and modulation of brain glutamate transporters by protein kinase C. J. Biol. Chem. 268, 27313-27317.

Chan, H., Zwingmann, C., Pannunzio, M., Butterworth, R. F., 2003. Effects of ammonia on high affinity glutamate uptake and glutamate transporter EAAT3 expression in cultured rat cerebellar granule cells. Neurochem. Int. 43, 137-146.

Chang, M. Y., Lee, S. H., Kim, J. H., Lee, K. H., Kim, Y. S., Son, H., Lee, Y. S., 2001. Protein kinase C-mediated functional regulation of dopamine transporter is not

achieved by direct phosphorylation of the dopamine transporter protein. J. Neurochem. 77, 754-761.

Dall'Asta, V., Gazzola, G. C., Franchi-Gazzola, R., Bussolati, O., Longo, N., Guidotti, G. G., 1983. Pathways of L-glutamic acid transport in cultured human fibroblasts. J. Biol. Chem. 258, 6371-6379.

Davis, K. E., Straff, D. J., Weinstein, E. A., Bannerman, P. G., Correale, D. M., Rothstein, J. D., Robinson, M. B., 1998. Multiple signaling pathways regulate cell surface expression and activity of the excitatory amino acid carrier 1 subtype of Glu transporter in C6 glioma. J. Neurosci. 18, 2475-2485.

Dolinska, M., Dybel, A., Zablocka, B., Albrecht, J., 2003. Glutamine transport in C6 glioma cells shows ASCT2 system characteristics. Neurochem. Int. 43, 501-507.

Dowd, L. A., Coyle, A. J., Rothstein, J. D., Pritchett, D. B., Robinson, M. B., 1996. Comparison of Na+-dependent glutamate transport activity in synaptosomes, C6 glioma, and Xenopus oocytes expressing excitatory amino acid carrier 1 (EAAC1). Mol. Pharmacol. 49, 465-473.

Dowd, L. A., Robinson, M. B., 1996. Rapid stimulation of EAAC1-mediated Na+-dependent L-glutamate transport activity in C6 glioma cells by phorbol ester. J. Neurochem. 67, 508-516.

Duan, S., Anderson, C. M., Stein, B. A., Swanson, R. A., 1999. Glutamate induces rapid upregulation of astrocyte glutamate transport and cell-surface expression of GLAST. J. Neurosci. 19, 10193-10200.

Forster, I. C., Traebert, M., Jankowski, M., Stange, G., Biber, J., Murer, H., 1999. Protein kinase C activators induce membrane retrieval of type II Na+-phosphate cotransporters expressed in Xenopus oocytes. J. Physiol. 517, 327-340.

Fournier, K. M., Gonzalez, M. I., Robinson, M. B., 2004. Rapid trafficking of the neuronal glutamate transporter, EAAC1: evidence for distinct trafficking pathways differentially regulated by protein kinase C and platelet-derived growth factor. J. Biol. Chem. 279, 34505-34513.

Franchi-Gazzola, R., Bussolati, O., Visigalli, R., Rotoli, B. M., Rossi, P. A., Guidotti, G. G., Gazzola, G. C., 1990. Phorbol esters stimulate the transport of anionic amino acids in cultured human fibroblasts. Biochem. Biophys. Res. Commun. 173, 1304-1310.

Franchi-Gazzola, R., Visigalli, R., Bussolati, O., Gazzola, G. C., 1996. Involvement of protein kinase Cepsilon in the stimulation of anionic amino acid transport in cultured human fibroblasts. J. Biol. Chem. 271, 26124-26130.

Gazzola, G. C., Dall'Asta, V., Bussolati, O., Makowske, M., Christensen, H. N., 1981. A stereoselective anomaly in dicarboxylic amino acid transport. J. Biol. Chem. 256, 6054-6059.

Gonzalez, M. I., Bannerman, P. G., Robinson, M. B., 2003. Phorbol myristate acetate-dependent interaction of protein kinase Calpha and the neuronal glutamate transporter EAAC1. J. Neurosci. 23, 5589-5593.

Gonzalez, M. I., Kazanietz, M. G., Robinson, M. B., 2002. Regulation of the neuronal glutamate transporter excitatory amino acid carrier-1 (EAAC1) by different protein kinase C subtypes. Mol. Pharmacol. 62, 901-910.

Guillet, B. A., Velly, L. J., Canolle, B., Masmejean, F. M., Nieoullon, A. L., Pisano, P., 2005. Differential regulation by protein kinases of activity and cell surface expression of glutamate transporters in neuron-enriched cultures. Neurochem. Int. 46, 337-346.

Kanai, Y., Hediger, M. A., 2004. The glutamate/neutral amino acid transporter family SLC1: molecular, physiological and pharmacological aspects. Pflugers Arch. 447, 469-479.

Mackenzie, B., Erickson, J. D., 2004. Sodium-coupled neutral amino acid (System N/A) transporters of the SLC38 gene family. Pflugers Arch. 447, 784-795.

Najimi, M., Maloteaux, J. M., Hermans, E., 2002. Cytoskeleton-related trafficking of the EAAC1 glutamate transporter after activation of the G(q/11)-coupled neurotensin receptor NTS1. FEBS Lett. 523, 224-228.

Najimi, M., Maloteaux, J. M., Hermans, E., 2005. Pertussis toxin-sensitive modulation of glutamate transport by endothelin-1 type A receptors in glioma cells. Biochim. Biophys. Acta 1668, 195-202.

Otis, T. S., Brasnjo, G., Dzubay, J. A., Pratap, M., 2004. Interactions between glutamate transporters and metabotropic glutamate receptors at excitatory synapses in the cerebellar cortex. Neurochem. Int. 45, 537-544.

Pajor, A. M., Sun, N., 1999. Protein kinase C-mediated regulation of the renal Na(+)/dicarboxylate cotransporter, NaDC-1. Biochim. Biophys. Acta 1420, 223-230.

Palos, T. P., Ramachandran, B., Boado, R., Howard, B. D., 1996. Rat C6 and human astrocytic tumor cells express a neuronal type of glutamate transporter. Brain Res. Mol. Brain Res. 37, 297-303.

Peterson, J. R., Mitchison, T. J., 2002. Small molecules, big impact: a history of chemical inhibitors and the cytoskeleton. Chem. Biol. 9, 1275-1285.

Robinson, M. B., 2002. Regulated trafficking of neurotransmitter transporters: common notes but different melodies. J. Neurochem. 80, 1-11.

Sakai, N., Kodama, N., Ohmori, S., Sasaki, K., Saito, N., 2000. Involvement of the actin cytoskeleton in the regulation of serotonin transporter (SET) activity: possible mechanism underlying SET regulation by protein kinase C. Neurochem. Int. 36, 567-579.

Schniepp, R., Kohler, K., Ladewig, T., Guenther, E., Henke, G., Palmada, M., Boehmer, C., Rothstein, J. D., Broer, S., Lang, F., 2004. Retinal colocalization and in vitro interaction of the glutamate transporter EAAT3 and the serum- and glucocorticoid-inducible kinase SGK1. Invest. Ophthalmol. Vis. Sci. 45, 1442-1449.

Sims, K. D., Straff, D. J., Robinson, M. B., 2000. Platelet-derived growth factor rapidly increases activity and cell surface expression of the EAAC1 subtype of glutamate transporter through activation of phosphatidylinositol 3-kinase. J. Biol. Chem. 275, 5228-5237.

Wolff, N. A., Thies, K., Kuhnke, N., Reid, G., Friedrich, B., Lang, F., Burckhardt, G., 2003. Protein kinase C activation downregulates human organic anion transporter 1-mediated transport through carrier internalization. J. Am. Soc. Nephrol. 14, 1959-1968.

Zafra, F., Aragon, C., Gimenez, C., 1994. Characteristics and regulation of proline transport in cultured glioblastoma cells. Biochem. J. 302, 675-680.

Legends to figures

Fig.1. Effect of latrunculin A and cytochalasin D on actin microfilaments. C6 cells, seeded on four-chamber coverslides, were incubated in normal growth medium (control, Panel A), or in medium supplemented with latrunculin A (0.6 μM, 15 min, panel B) or with cytochalasin D (5 μM, 30 min, panel C). After the treatments, cells were fixed and stained with phalloidin and propidium iodide (see Experimental Procedures). Single confocal sections of representative fields are shown. In the acquired images, the intensity of phalloidin signal is rendered in a green scale, while the propidium signal is rendered in a scale of reds (see the palettes in Panel A). Bar = 10 μm, for all Panels.

Fig. 2. Effects of latrunculin A on EAAT3 transport activity in C6 glioma cells. Cells were seeded at a density of 10.000 cells/well in 96-well multiwell plates. Panel A. After 2d, the medium was supplemented with 0.6 μM latrunculin A for the indicated times. Aspartate transport was then determined as described in Experimental Procedures. ***$P < 0.001$ as assessed with one way ANOVA. Panel B. Cells were grown as described in Panel A. At time 0, cells were incubated in the presence of 0.6 μM latrunculin A (LA) for 15 min. After this period, medium was replaced with toxin-free fresh growth medium and incubation prolonged for 60 min. Aspartate transport was measured at the indicated times. Panel C. Cells were incubated for 15 min in the absence or in the presence of 0.6 μM latrunculin A as indicated. The uptake of a range of L-Asp concentrations from 0.4 μM to 200 μM was then determined. The diffusive component, calculated from the transport of 2 mM L-Asp and comparable in control and treated cells, was subtracted from uptake values before data fitting. Lines represent the best fit of transport data to Eq. 1 (see Methods). Goodness of fit was assessed from determination coefficients that were 0.977 and 0.971 for transport in the absence or in the presence of latrunculin, respectively. For transport parameters,

see text. For the three Panels data are means of five independent determinations with S.D. shown when greater than the size of the point.

Fig. 3. Latrunculin A does not prevent EAAT3 stimulation by PDBu. Cells were incubated for the indicated times with 0.6 μM latrunculin A. At the end of the incubation L-Asp transport was determined as described in Experimental Procedures. Ten minutes before the transport assay medium was supplemented either with vehicle (empty bars) or with 150 nM PDBu (solid bars). Data are means of five independent determinations with S.D, shown. *P < 0.05, **P < 0.01, compared with cells non treated with either latrunculin A or PDBu; $^{\#}P$ < 0.01, compared with cells not treated with PDBu incubated for the same period with latrunculin.

Fig. 4. Effects of PDBu and latrunculin A on EAAT3 membrane expression. Panel A. Western blot analysis of EAAT3 protein (upper part) and β-tubulin (lower part) expression was performed on biotinylated membrane proteins (left), intracellular proteins (middle), or total cell lysates (right) of C6 cells treated with latrunculin A (0.6 μM, 15 min) and/or PDBu (150 nM, 10 min), as indicated. Panel B. Densitometric analysis of EAAT3 expression shown in Panel A. The experiment was performed two times with comparable results.

Fig. 5. PDBu pre-incubation prevents inhibition of EAAT3 activity by latrunculin A. Cells were incubated for 10 min in the absence (empty bars) or in the presence (solid bars) of 150 nM PDBu and, in the last 5 min of PDBu treatment, with latrunculin A (0.6 μM). At the end of this period, L-Asp transport was measured. Data are means of five independent determinations with S.D. shown. **P < 0.01, compared with the corresponding control cells non treated with latrunculin A.

Fig. 6. Effects of cytochalasin D on EAAT3 activity and membrane expression. Panel A. Cells were incubated for the indicated times with 5 μM cytochalasin D. At the end of the incubation L-Asp transport was determined as described in Experimental Procedures. Ten minutes before the transport assay medium was supplemented either with vehicle (empty bars) or with 150 nM PDBu (solid bars). Data are means of five independent determinations with SD shown. $^{**}P < 0.01$, compared with cells non treated with either cytochalasin D or PDBu; $^{\#}P < 0.01$, compared with cells not treated with PDBu incubated for the same period with cytochalasin D. Panel B. Western blot analysis of EAAT3 protein in the biotinylated fraction of C6 cells treated with cytochalasin D for 30 min and incubated for the last 10 min of treatment in the absence or in the presence of 150 nM PDBu, as indicated. The experiment was repeated twice with comparable results.

Fig. 7. Dose-dependence of jasplakinolide effects on EAAT3 activity. Cells were incubated for 30 min with the indicated concentrations of jasplakinolide in the absence (empty bars) or in the presence (solid bars) of 150 nM PDBu in the last 10 min of incubation. At the end of the incubation, L-Asp transport was determined as described in Experimental Procedures. Data are means of five independent determinations with SD shown. $^{**}P < 0.01$, compared with control cells not treated with either jasplakinolide or PDBu; $^{\#}P < 0.01$, compared with cells treated with PDBu but not with jasplaskinolide.

Fig. 8. Effects of latrunculin A and PDBu on the transport of neutral amino acids. C6 cells were incubated for 10 min in the absence or in the presence of 0.6 μM latrunculin A (LA) and/or 150 nM PDBu, as indicated. At the end of this period, the uptakes of L-proline (0.1 mM, 4 μCi/ml), L-threonine (0.05 mM, 3 μCi/ml), and L-glutamine (0.1 mM, 3 μCi/ml) were measured in 1-min assays. For the three panels, data are means of five independent determinations with SD shown. $^{*}P < 0.05$, compared with cells not treated with PDBu.

Fig. 9. Effect of microtubule toxins on EAAT3 activity in C6 cells. L-Aspartate uptake was measured in C6 cells after a 30 min-treatment with 1 μM colchicine or 1 μM paclitaxel in the absence (empty bars) or in the presence (solid bars) of 150 nM PDBu in the last 10 min of incubation. Data are means of five independent determinations with SD shown. *P < 0.05, **P < 0.01, compared with cells not treated with either latrunculin A or PDBu.

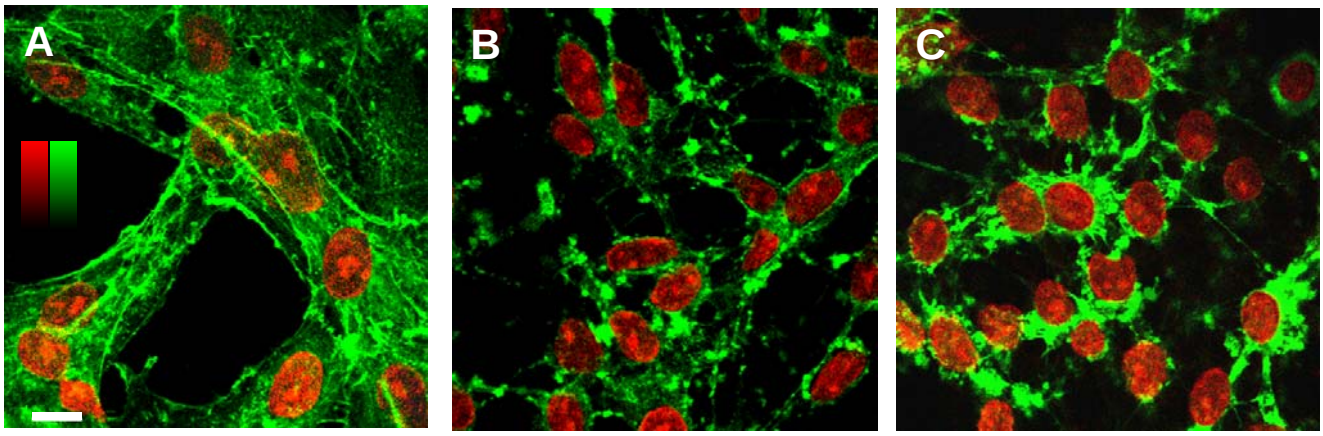


Figure1

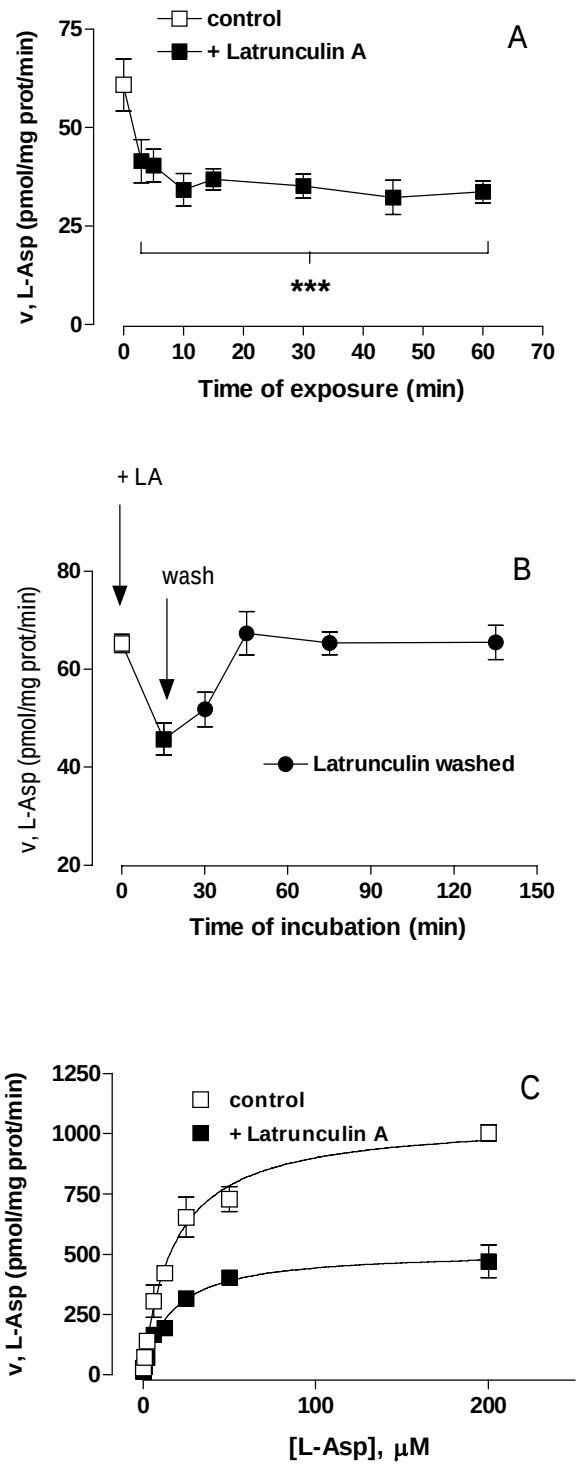
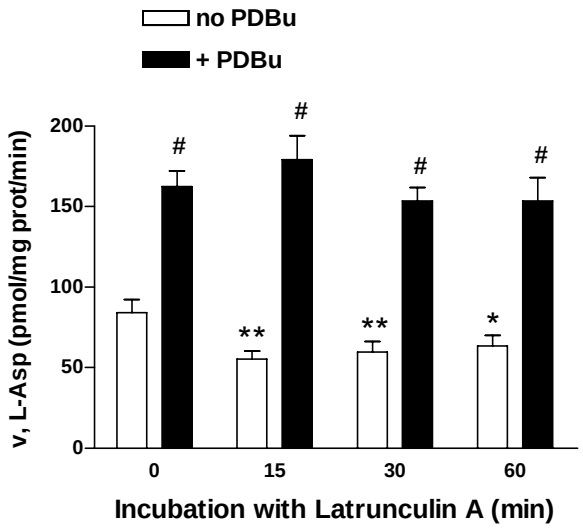

control
+ Latrunculin A
A
v, L-Asp (pmol/mg prot/min)
75
50
25
0
***
0
10
20
30
40
50
60
70
Time of exposure (min)
+ LA
wash
B
80
60
40
20
Latrunculin washed
0
30
60
90
120
150
Time of incubation (min)
C
1250
1000
750
500
250
0
100
200
[L-Asp], µM

Figure 2


no PDBu
+ PDBu
v, L-Asp (pmol/mg prot/min)
200
150
100
50
0
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*
0
15
30
60
Incubation with Latrunculin A (min)

Figure 3

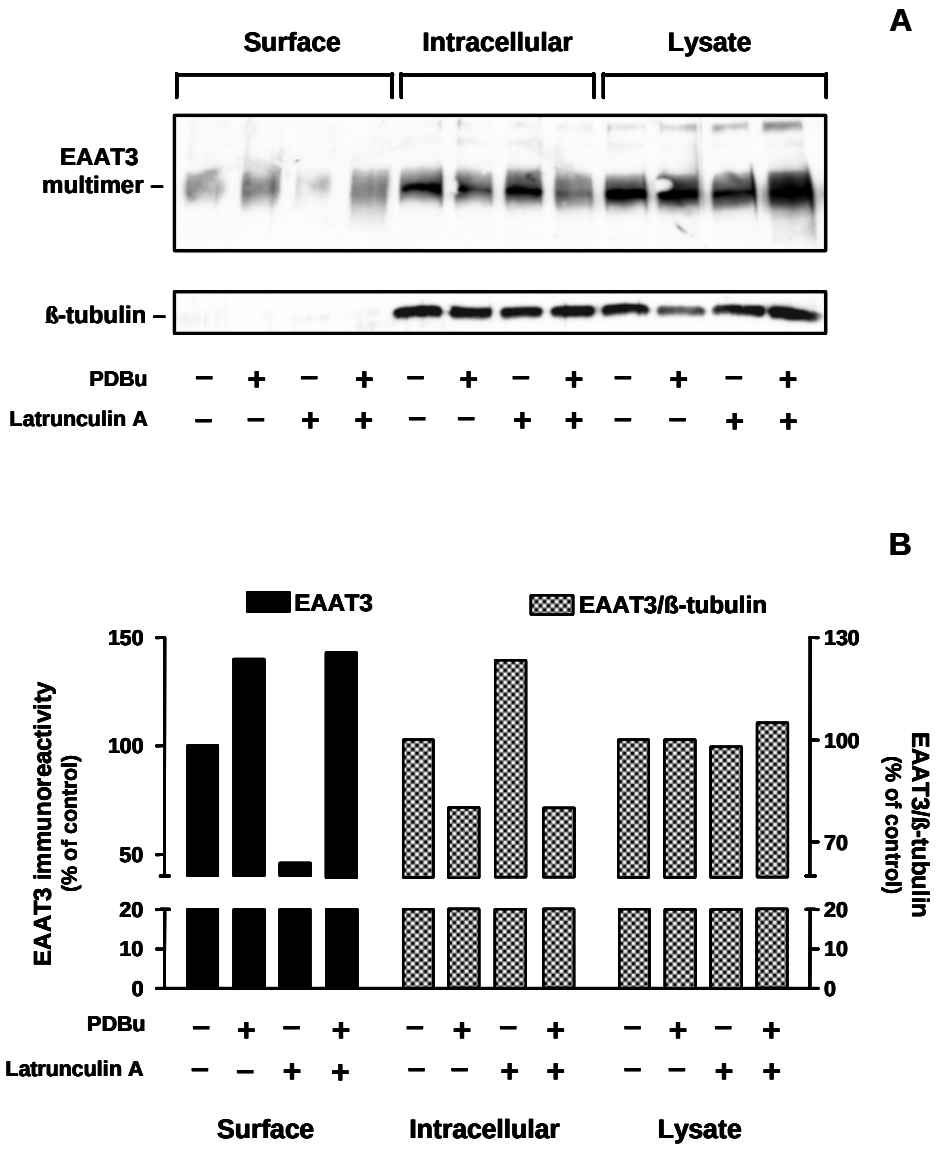

A
Surface
Intracellular
Lysate
EAAT3
multimer –
ß-tubulin –
PDBu
Latrunculin A
B
EAAT3
EAAT3/ß-tubulin
EAAT3 immunoreactivity
(% of control)
EAAT3/ß-tubulin
(% of control)
150
100
50
20
10
0
130
100
70
20
10
0
PDBu
Latrunculin A
Surface
Intracellular
Lysate

Figure 4

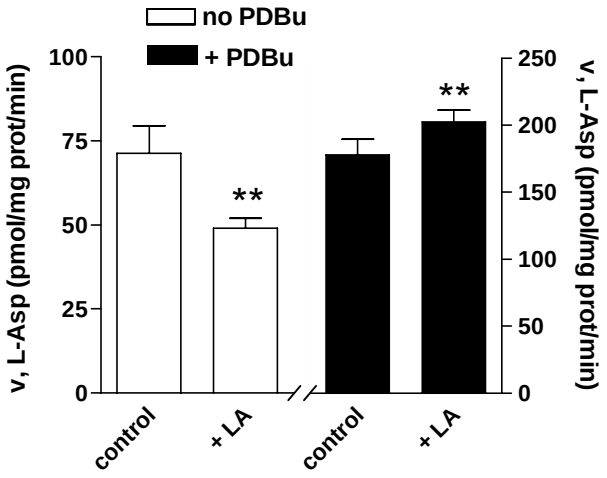
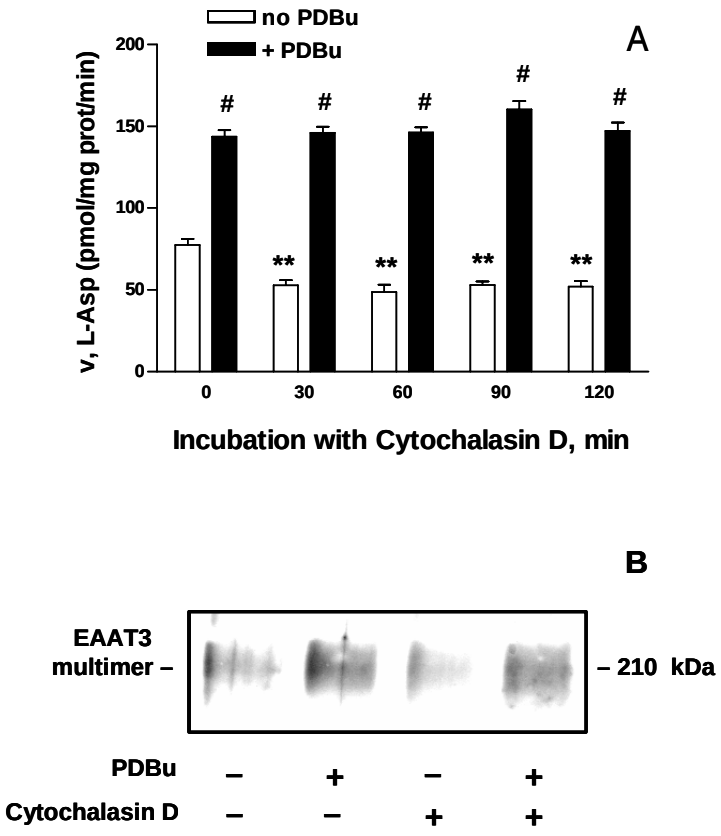

no PDBu
+ PDBu
v, L-Asp (pmol/mg prot/min)
v, L-Asp (pmol/mg prot/min)
100
75
50
25
0
250
200
150
100
50
0
**
**
control
+ LA
control
+ LA

Figure 5


no PDBu
+ PDBu
A
v, L-Asp (pmol/mg prot/min)
200
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0
30
60
90
120
Incubation with Cytochalasin D, min
B
EAAT3
multimer –
– 210 kDa
PDBu
Cytochalasin D
–
+

Figure 6

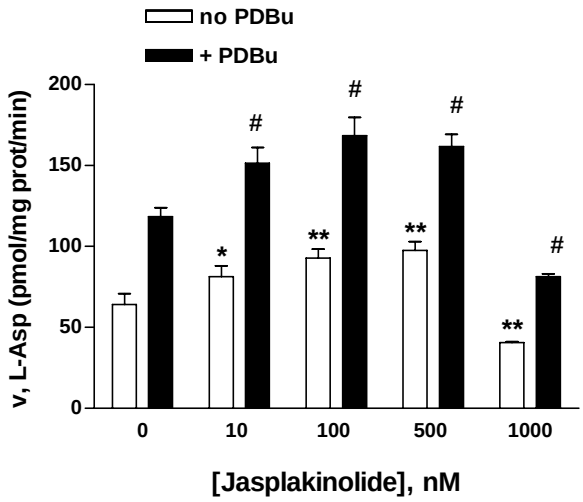
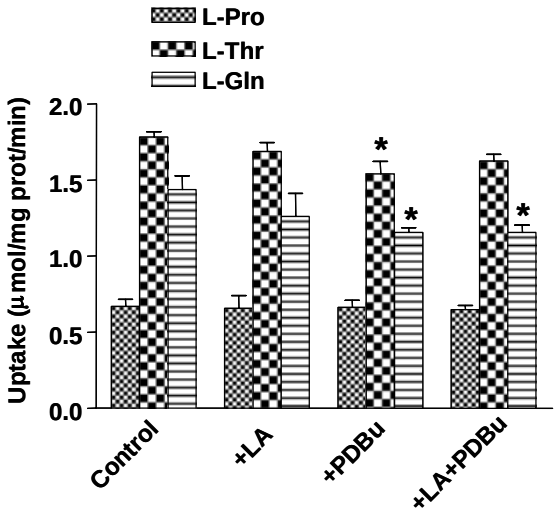

no PDBu
+ PDBu
v, L-Asp (pmol/mg prot/min)
200
150
100
50
0
*
**
**
**
#
#
#
#
0
10
100
500
1000
[Jasplakinolide], nM

Figure 7


L-Pro
L-Thr
L-Gln
Uptake (µmol/mg prot/min)
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1.5
1.0
0.5
0.0
*
*
*
Control
+LA
+PDBu
+LA+PDBu

Figure 8

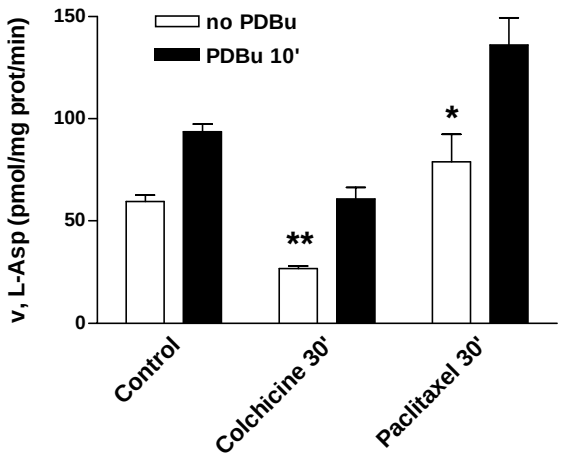
150
100
50
0
v, L-Asp (pmol/mg prot/min)
no PDBu
PDBu 10'
**
*
Control
Colchicine 30'
Paclitaxel 30'

Figure 9

In summary, in this first paper (Bianchi et al., 2006) we have found that actin-perturbing agents, such as latrunculin A and cytochalasin D, decrease glutamate transport and EAAC1 membrane expression in C6 cells, suggesting that actin cytoskeleton is involved in EAAC1 trafficking. Moreover, we demonstrated that actin disorganization does not interfere with PKC-dependent stimulation of EAAC1 membrane expression and glutamate transport stimulation, suggesting that the redistribution of the carrier promoted by PKC activation is cytoskeleton-independent. However, no hints were obtained about the possible cytoskeletal partners of EAAC1.

Studies carried out in other cell models demonstrated that the molecular sorting of Na/K ATPase is affected by the Rho-associated kinase (ROCK), a protein which regulates actin dynamics (Lecuona et al., 2003). This finding made us to focus on actin-linked proteins, able to influence the organization status of actin microfilaments, as possible partners of EAAT3/EAAC1. The hypothesis was further supported when we observed that, in primary cultures of rat dermal fibroblasts, the transport activity for aspartate, due to EAATs, was significantly affected by the expression of mutant forms of adducin (see below).

Adducin is an ubiquitous heteromeric protein containing a COOH-terminal myristoylated alanine-rich PKC substrate (MARCKS)-related domain originally described in red blood cells where it caps and preferentially recruits spectrin to the fast-growing ends of actin filaments. The basic MARCKS-related domain, present in alpha, beta, and gamma adducin subunits, binds calmodulin and contains the major phosphorylation site for protein kinase C (PKC) in multiple cell types, including neurons (Matsuoka et al., 1998, 2000). Interestingly high levels of adducin were detected in the dendritic spines of cultured hippocampal neurons and its phosphorylation seems to be linked to the synaptic plasticity following actin remodelling. Adducin is also abundant in platelets where its phosphorylation leads to platelet activation and possibly adducin degradation (Gilligan et al., 2002; Barkalow et al., 2003).

The protein has been investigated in depth since adducin polymorphisms have been strongly linked to some forms of essential hypertension in both humans and rats. In particular, it has been observed that congenic rats for the mutant F316Y α-adducin develop an increase in blood pressure associated with faster kidney tubular ion transport (Tripodi et al., 1996). The possible mechanism underlying this phenomenon has been recently described. Efendieve et al. showed indeed that the expression of the hypertensive rat or human variant of the mutant α-adducin into normal renal epithelial cells mimics the hypertensive phenotype causing a marked stimulation of Na/K-ATPase activity due to the impaired Na/K-ATPase endocytosis via AP-2 protein. (Efendiev et al., 2004; Torielli et al., 2008).

These observations suggest that adducin may influence the membrane expression and activity of sodium-dependent transporters. Indeed, preliminary transport experiments carried out on fibroblast primary cultures from congenic rats, showed that F316Y α-adducin reduced aspartate uptake through changes in the V_{max} of a high affinity transporter, presumably corresponding to a carrier of the EAAT family. The transporter exhibited the operational and kinetic features of system $X^-_{AG,}$, as previously described many years ago in mesenchymal cells by our laboratory (Gazzola et al., 1981; Dall'Asta et al., 1983; Franchi-Gazzola et al., 1996). Western blot experiments showed the presence of both EAAT3/EAAC1 and EAAT1/GLAST in rat fibroblasts. However, the expression levels of the two transporters were so low that clear cut conclusions on the carrier affected by the expression of α-adducin mutants could not be drawn.

Therefore, we decided to ascertain the possibility that adducin interacts with EAAT3/EAAC1 performing both colocalization and co-immunoprecipitation experiments in C6 cells (Bianchi et al., submitted, 2009a).

EAAC1 GLUTAMATE TRANSPORTER INTERACTS WITH THE ACTIN-BINDING PROTEIN α-ADDUCIN IN C6 GLIOMA CELLS

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Abbreviations ATRA, all-*trans*-retinoic acid; DMEM, Dulbecco's modified Eagle Medium; EAAC1, Excitatory Amino Acid Carrier 1; EAAT, Excitatory Amino Acid Transporter; FBS, Fetal Bovine Serum; PDBu, Phorbol 12,13-dibutyrate.

Abstract

The ubiquitous EAAC1 transporter belongs to the family of the Na^+-dependent glutamate carriers expressed mainly in the CNS. Recent studies reported the association between defective function of EAAC1 and neurologic disease. However, the mechanisms underlying EAAC1 regulation are not yet fully understood. We have reported that in C6 glioma cell line both the activity and membrane targeting of EAAC1 require the integrity of actin cytoskeleton. Here we show that, in the same model, EAAC1 partially co-localizes with actin filaments, at the level of cell processes, as well as with the actin binding protein adducin in the perinuclear zone. Co-immunoprecipitation experiments confirm an interaction of EAAC1 and adducin. The co-localization and the co-immunoprecipitation are still detectable after cell treatment with phorbol esters, a condition that leads to a PKC-dependent increase of EAAC1 expression on the membraney and to the phosphorylation of adducin. The amount of adducin co-immunoprecipitated with EAAC1 increases after the treatment of C6 cells with retinoic acid, a differentiating agent that induces an overexpression of both EAAC1 mRNA and protein in this cell model. Moreover, in clones of C6 cells transfected with a HA-tagged adducin, bands of EAAC1 immunoprecipitated by an anti-HA antiserum were proportional to EAAC1 expression. These results suggest the existence of a pool of EAAC1 transporters interacting in a PKC-insensitive manner with the actin binding protein adducin.

Key words

Actin - Slc1a1 - EAAT3 - Cytoskeleton – Retinoic acid

EAAC1 (EAAT3 in humans) is the main transporter for excitatory amino acids of neurons although its expression is ubiquitous [1]. It is believed that this transporter plays an important, although only partially characterized, role in the regulation of glutamatergic synapses and in the preservation of neuronal integrity [2]. Alterations in EAAC1 expression and/or activity have been implied in neuroprotection [3-6] and involved in several disorders, such as epilepsy [7], ischemic stroke [8, 9], schizophrenia [9], and obsessive-compulsive disorder [10].

Although the expression of the transporter in the brain is mostly concentrated in neurons, a widely used cell model in studies of EAAC1 regulation is represented by rat glioma C6 cells. C6 cells are particularly useful since the only EAAT carrier expressed at high levels in this model is EAAC1 [11, 12], thus rendering functional studies simpler. In C6 cells it has been demonstrated that phorbol esters, through PKC activation, inhibit EAAC1 internalization, thus causing an increase in the membrane expression of the transporter and a stimulation of glutamate transport [13]. More recently, members of the SNARE family have been involved in EAAC1 trafficking that would occur through a pool of chlatrin-coated vesicles [14]. Interestingly, in vitro and in vivo have indicated that, at variance with other EAAT transporters, EAAC1 localization is mostly intracellular [15-17], suggesting that changes in transporter redistribution may be of great importance its regulation.

In a recent contribution [18], we found that actin-perturbing agents, such as latrunculin A and cytochalasin D, decrease glutamate transport and EAAC1 membrane expression in C6 cells, suggesting that actin cytoskeleton may be involved in EAAC1 trafficking. However, actin disorganization did not interfere with PKC-dependent stimulation of EAAC1 membrane expression and activity, suggesting that the redistribution of the carrier promoted by PKC activation is cytoskeleton-independent [18]. No hints were obtained in that study about

possible cytoskeletal partners of EAAC1. However, cytoskeletal perturbations modify the trafficking of other EAAT transporters [19].

Adducin is a ubiquitously expressed, oligomeric, cytoskeletal protein that is an in vivo substrate for protein kinase C (PKC) and Rho-associated kinase in hippocampal neurons, platelets, and growth cones of axons [20]. In the cytoskeleton, adducin is present as a heterotetramer formed by two α subunits non covalently linked to two β or, alternatively, to two γ subunits depending on the cell type. Besides other cytoskeletal proteins, known partners of adducin are a putative nuclear receptor [21] and a PKC isozyme involved in the regulation of glucose transporter trafficking [22]. Results obtained in reconstituted systems have suggested the existence of an interaction between α-adducin and Na(+)-K(+)-ATPase [23]. More recently, α-adducin has been found to interact with AP2 protein and to influence Na,K-ATPase trafficking [24], pointing to the possibility that the cytoskeletal protein may regulate the activity of other membrane transporters. These data prompted us to assess the existence of an interaction between adducin and EAAC1 transporter. Here we demonstrate that in C6 glioma cells a partial co-localization exists between α-adducin and the transporter. The interaction between the two proteins has been also confirmed by co-immunoprecipitation experiments.

Experimental procedures

Cells

The rat central nervous system-derived C6 glioma cells were obtained from American Type Culture Collection (Rockville, MD). Cells were grown at 37°C and 5% CO_2 in Dulbecco's modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 4 mM glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin. Cells were used for less than 10 passages from thawing with no apparent change in morphology or sensitivity to treatments.

Experimental treatments

Drugs were obtained from Sigma-Aldrich, Milan, Italy, unless otherwise specified. Final and stock concentrations were 0.6 µM and 300 µM (in DMSO) for latrunculin A; 150 nM and 1 mM (in ethanol) for phorbol 12,13-dibutyrate (PDBu); 10 µM and 10 mM (in DMSO) for all *trans*-retinoic acid (ATRA). Vehicles were added to control cells at the maximal concentration used for drug treatments.

Immunofluorescence

To study the subcellular distribution of EAAC1 transporters and their relationship with actin cytoskeleton, a triple immunofluorescence staining was performed. For both EAAC1 and α-adducin we adopted an immunostaining approach, while actin was detected using labelled phalloidin. A multi step approach was adopted in which EAAC1 staining was completed first, followed by adducin labelling and, as the last step, actin detection. Briefly, C6 cells were seeded on coverslips at a density of $20 \cdot 10^3$ cells/cm^2 and treated after 48h. After the experimental treatments, cells were rinsed in PBS and fixed for 10 min in 3.7%

paraformaldehyde in PBS. After two further rinses, cells were permeabilized with a 7-min incubation in 0.1% Triton X-100 in PBS. Cells were then incubated for 1h in blocking solution (2% of BSA in PBS) followed by an incubation with anti-EAAC1 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:50), two washes in PBS, and an additional 1h-incubation Alexa Fluor 543 anti-rabbit antibody (Invitrogen, Paisley, UK,, 1:400). After one rinse, cells were incubated with a monoclonal anti-α-adducin antibody (raised by Prassis SpA, Milan, Italy; 1:40) and then to Alexa Fluor 488 anti-mouse antibody (Invitrogen, 1:400). After two further rinses, actin was stained with a 20 min incubation in the presence of AlexaFluor 633 phalloidin (Invitrogen, 4U/ml). In the experiments in which only EAAC1 and actin were visualized (see Fig. 1), AlexaFluor 488 phalloidin (Invitrogen, 4U/ml) was used.

The coverslips were then mounted and analyzed with a Zeiss® 510 LSM Meta confocal microscope using a multi-track detection system. A 63X (NA 1.4 oil) objective was used. EAAC1 signal is rendered with a scale of reds (from 0 to 256), while actin is rendered in a scale of greens (from 0 to 256). The LSM 5® software, Version 3.5, was used to analyze the images so as to obtain the number of positive pixels and the mean signal intensity.

Co-immunoprecipitation and Western Blot

Cultures of C6 cells were grown to subconfluence on 10-cm^2 tissue culture plates. After the experimental treatment, cells were rinsed twice in PBS and incubated in 800 µl of RIPA buffer (10 mM phosphate buffer, pH 7.5, 80 mM NaCl, 1 EDTA, 0.1% Triton 100X, 1 mM sodium orthovanadate) containing a cocktail of protease inhibitors (Roche SpA, Monza, Italy) for 1h at 4°C. After solubilization, cell lysates were collected in eppendorf tubes and centrifuged at 14000 *g* for 30 min. The supernatants were transferred into new tubes, quantification of proteins was performed with Biorad assay method, and protein content was adjusted to 600

μg/ml. Supernatants were then precleared with 80 μl of IP matrix from ExactaCruz kit (Santa Cruz). After 1h of incubation with gently shaking, samples were spinned and the supernatants were transferred into new tubes to obtain pre-cleared lysate. In parallel, 10 μg of anti-α-adducin or anti-EAAC1 antiserum in 500 μl of PBS were combined with 80 μl of beads to form the immunomatrix. In the experiments performed with C6 cells expressing HA-adducin, the immunomatrix was made with the monoclonal HA-11 antibody, raised against the HA tag (Covance, Emeryville, CA). After 4h at 4°C, the immunomatrix was centrifuged and the pellet was washed 2 times with PBS. 1ml of precleared total lysate was then added to each pellet and incubated overnight at 4°C. The samples were then centrifuged and the immunoprecipitates separated from the supernatant, rinsed twice with PBS, suspended in 50 μl of 2X sample buffer, and boiled for 5 min. SDS-PAGE of the immunoprecipitates was then performed, with 35-μg-aliquots of the total cell lysates and of the supernatants of the immunoprecipitation mixtures, dissolved in 4X sample buffer, running in parallel. After blotting, the membranes were exposed overnight to anti-EAAC1 polyclonal antiserum (1:2000), anti-α-adducin monoclonal antibody (1:2000), or anti-HA antibody (1:1000), diluted in blocking solution. After washing, the blots were exposed to HRP-anti-rabbit detection reagent (Santa Cruz, 1:5000) and/or HRP-anti-mouse detection reagent (Santa Cruz, 1:5000) in blocking solution for 1h. The membranes were washed and the immunoreactivity visualized with enhanced chemiluminescence (Amersham Pharmacia Biotech Italia, Milan, Italy).

For the detection of phosphorylated α-adducin, aliquots of 35 μg of total cell lysates and a monoclonal anti-Ser724-phospho-α-adducin antibody (Upstate, Lake Placid, NY, USA, 1:1000) were used.

Stable C6 Cell Transfection

The vector for hemagglutinin (HA)-tagged rat α-adducin was obtained by cDNA amplification and subcloning into the *Nhe*I–*Xho*I sites of the pCMVneoHA vector [24]. Rat HA-α-adducin was transfected into C6 cells with a Nucleofector II (Amaxa Inc., Gaithersburg, MD), following the instructions of the manufacturer. Stable transfectants were selected by addition of G418 sulphate and subcultured by ring isolation, obtaining 30 clones for further characterization.

EAAC1 expression and activity

Total RNA was isolated with RNeasy Mini Kit® (Qiagen S.p.a., Milan, Italy). After reverse transcription, 25 ng of cDNA from each sample was amplified in a total volume of 25 _l with 2X Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen), along with the following forward and reverse primers (5 pmol each): 5' CGA CTT GCC GTA CCT GGA CT 3' and 5' GCC CAC GGG ACT CAA CAC GA 3', for Slc1a1; 5' AGC CTC AAG ATC ATC AGC AAT G 3= and 5' CAC GAT ACC AAA GTT GTC ATG GA 3' for Gapdh. All the set of primers were designed with the help of Primer 3 program [25] according to the known sequences reported in GenBank.

Quantitative PCR was performed in a 36 well Rotor Gene™ 3000, version 5.0.60 (Corbett Research, Rotor-Gene™ 3000, version 5.0.60, Mortlake, Australia). For all the probands each cycle consisted of a denaturation step at 95 °C for 15 s, followed by separate annealing (30 s) and extension (15 s) steps at a temperature characteristic for each proband. Fluorescence was monitored at the end of each extension step. A no-template, noreverse transcriptase control was included in each experiment. At the end of the amplification cycles a melting curve analysis was added. The analysis of the data was made according to the

relative standard curve method [26]. qRT-PCR data were expressed as the ratio between Slc1a1 mRNA and Gapdh mRNA.

EAAC1 activity was assessed from the initial influx of D-aspartate [27]. Briefly, C6 cells, seeded at a density of $10 \cdot 10^3$ cells/well were washed twice in a modified EBSS (Earle's balanced salt solution, buffered with 20 mM Hepes–NaOH at pH 7.4) and incubated in the same saline solution, supplemented with D-[3H]aspartate (1 μM, 2 μCi/ml) for 1 min. At the end of the assay, multiwell dishes were washed twice with ice-cold urea (300 mM) and cell monolayers were extracted with 50 μl of absolute ethanol. The extracts were added to 200 μl of scintillation fluid and counted with a Wallac Trilux2 liquid scintillation spectrometer(Perkin-Elmer, Boston, MA, USA). Cell proteins were determined directly in the well with a modified Lowry procedure and measured with a Wallac Victor2 Multilabel Counter (Perkin-Elmer).

Materials

Serum and culture medium were obtained from Celbio Euroclone (Milan, Italy). D-[2,3-^{3}H]aspartic acid (12 Ci/mmol) was obtained from Perkin Elmer (Monza, Italy). Sigma was the source of the other chemicals whenever not stated otherwise.

Results

EAAC1 subcellular distribution and partial co-localization with actin and adducin

Although C6 glioma have been widely employed in studies on EAAC1 expression and regulation, very few immunocytochemical data have been reported on this model. The immunofluorescence analysis of EAAC1 and actin cytoskeleton shown in Fig. 1 demonstrates that C6 cultures exhibited a markedly heterogeneous pattern of transporter expression (Panel A). While high expression levels were observed in cells with round bodies and two well distinguished processes (round cells according to Ohira et al. [28]), low to intermediate expression levels were detected in more spread cells (called spindle or flattened [28]). In all the cells EAAC1 appeared mostly intracellular and concentrated in vesicle-like structures that tended to aggregate in the perinuclear region. A strong positivity was observed in cell processes, in particular at their extremities, where clusters of actin-EAAC1 co-localization were detected (see Fig. 1, Panel C, inset). However, most of EAAC1 signal was not co-localized with actin.

Fig. 2 reports the immunocytochemical analysis of actin (Panel A) and α-adducin expression (Panel B) in C6 cells. These cells had a diffuse expression of α-adducin, with a low nuclear signal and peripheral reinforcements in some cells. As expected for a cytoskeletal protein that binds to barbed ends of actin filaments and modifies their organization (Matsuoka et al., 2000), co-localization with actin was evident in the focal adhesions and at the level of some cortical actin fibers (Panel C). However, α-adducin was only incompletely co-localized with actin and is particularly abundant in the perinuclear region.

When EAAC1, α-adducin and actin were simultaneously observed (Figs. 3-5), areas of α-adducin-actin and EAAC1-actin co-localization were still clearly detectable, while very limited co-localization spots of the three proteins were observed only in some process tips of round

cells (Fig.3, Panel D). In contrast, more diffuse areas of EAAC1-α-adducin co-localization were observed in perinuclear vesicle-like formations of spread cells (Figs. 3-5, Panel D). However, the co-localization was not complete and distinct EAAC1 and α-adducin signals were still clearly detectable.

Figure 6 shows the effects on EAAC1 distribution of a 15-min incubation of C6 cells with phorbol esters, an experimental treatment that produces the maximal PKC-dependent increase in EAAC1 activity [29]. The effects of the phorbol were particularly evident in some spread cells with intermediate levels of expression of the transporter. In these cells, EAAC1 signal became evidently marginalized (Panel A), while the α-adducin signal is not significantly modified (Panel B). In responsive cells, the phorbol had dramatic effects on actin cytoskeleton that was markedly reorganized (Panel C): mostly stress fibers appeared disorganized and clusters of actin formed at the periphery of the cell resembling the podosome-like structures observed in other cell models [30]. In PDBu-treated cells areas of partial co-localization between EAAC1 and α-adducin were detectable in the perinuclear region. On the contrary, well evident zones of co-localization between EAAC1 and actin were particularly evident at the periphery of the phorbol-sensitive cells.

Although we did not detect any evident change in α-adducin signal in phorbol-treated C6 cells, it was demonstrated in other cell models that PKC-dependent phosphorylation of α-adducin promotes its calpain-dependent degradation [31]. To verify the effects of PKC-stimulation under our conditions, we performed a Western analysis of α-adducin as well as of its phosphorylated form, in control and PDBu-treated cells. The results indicate that PKC-dependent phosphorylation of α-adducin was a rapid process, already detected after 5 min, maximal after 15 min of treatment, and persisting for at least 60 min (results not shown). However, consistently with the immunocytochemical data (Fig. 4 panel B), no significant

decrease of total α-adducin was observed in cells treated with PDBu for 15 min (results not shown).

Partial co-immunoprecipitation of EAAC1 and adducin

To determine if EAAC1 transporter forms complexes with α-adducin in C6 cells, anti-EAAC1 antibodies were used for immunoprecipitation and α-adducin immunoreactivity was studied with WB in immunoprecipitates (Fig. 5, Panel A). The results indicate that a faint band of co-immunoprecipitation was observed either in control cells or in cells treated with 150 nM PDBu for 15 min. Since both EAAC1 [29, 32, 33] and α-adducin [34, 35] are substrates of PKC, this result indicates that the phosphorylation of the two proteins does not affect their interaction. However, the α-adducin bands seemed specific since no band was detectable in negative controls (Fig. 5, Panel A, Beads). To test the interaction between α-adducin and EAAC1 with the complementary antibodies, we used anti-α-adducin antibodies to immunoprecipitate C6 lysates and searched for EAAC1 immunoreactivity with Western Blot. The results (not shown) confirm that a small fraction of the transporter was co-immunoprecipitated with the cytoskeletal protein. To test if the interaction between α-adducin and EAAC1 was dependent on the status of organization of actin microfilaments, a co-immunoprecipitation experiment was performed after cell pre-treatment with latrunculin A, a toxin that disorganizes actin microfilaments increasing the abundance of G actin [36]. C6 cells treated for 15 min with the toxin, although exhibiting profound morphological changes [18], still show a faint band of α-adducin co-immunoprecipitated with EAAC1 (Panels C and D), indicating that the relationship between the two proteins is not significantly influenced by the ratio F actin/G actin.

Effect of ATRA on EAAC1-adducin co-immunoprecipitation

We have recently reported that C6 glioma cells differentiated by ATRA induce EAAC1 mRNA and, consequently, exhibit a markedly enhanced expression of the carrier protein without significant changes in its distribution [27]. In the experiment shown in Fig. 8 we have tested if EAAC1 induction affects the interaction of the transporter with α-adducin. Although the levels of the cytoskeletal protein were comparable in control and in cells treated with ATRA for four days (Panel A), a larger amount of α-adducin was co-immunoprecipitated with EAAC1 in ATRA-treated than in control cells. As expected, a huge increase of EAAC1 protein was observed in total cell lysate (Panel B).

Transfection of C6 cells with HA-adducin

Fourteen C6 clones expressing hemagglutinin (HA)-tagged adducin were characterized for the expression of endogenous and tagged adducin as well as of EAAC1 (Fig. 9, panel A). While, as expected, the expression of the transgene (HA-ADD) was highly heterogeneous, also the levels of endogenous adducin (ADD) and EAAC1 were extremely variable from clone to clone. Although we did not investigate further the origin of this heterogeneity, we presume that, at least for EAAC1, it is independent from the transfection procedure, since the parental C6 population is highly heterogeneous as far as the expression of the transporter is concerned [27]. Two clones, 11 and 21, were selected for further characterization, because they both expressed sizable levels of HA-adducin and were endowed with very different EAAC1 levels. This different expression had clearcut consequences, with aspartate transport, respectively, 5-fold lower and 3-fold higher than values recorded in parental, non transfected cells (Panel B). Different transport activities also corresponded to roughly proportional levels of Slc1a1 mRNA (Panel C). A co.immunoprecipitation experiment, performed in Clones 11

and 21 (Panel D), indicated that the lysates of both populations yielded two EAAC1 bands when immunoprecipitated with anti-HA-adducin antiserum. Although the amount of tagged adducin contained in the precipitates was even smaller in clone 11 than in clone 21, the EAAC1 band was much more evident in clone 21.

Discussion

Although several reports have described regulatory mechanisms of glutamate transport based on transporters trafficking [13, 32, 33], the information about cytoskeletal involvement in these regulations is still far from complete. However, all the scaffolding proteins linked to EAAT carriers, such as Glu transporters-associated proteins (GTRAPs) and Ajuba, have been related to actin cytoskeleton [37-40]. Moreover, previous results from our laboratory have pointed to an interaction between EAAC1 and the actin cytoskeleton [18], although the regulation by PKC-dependent phosphorylation seems cytoskeleton-independent and possible cytoskeletal partners of EAAC1 are still elusive. In this contribution we provide evidence for a partial co-localization of EAAC1 with actin and, moreover, present experimental data that point to α-adducin as a partner of EAAC1. These conclusions are based both on immunocytochemistry and co-immunoprecipitation experiments.

The immunocytochemical analysis of C6 cells indicates an heterogeneous expression level of EAAC1 that correlates with distinct morphologies, previously described in this cell line and characterized by a peculiar cytoskeletal organization [28]. The cells with the highest EAAC1 expression are elements with round bodies and well defined processes (round cells), which are characterized by a relatively poor abundance of stress fibers and low activities of RhoA and ROCK [28]. Cells with a more flattened morphology and a more organized actin cytoskeleton have a lower expression of EAAC1. Interestingly, some of these cells respond very markedly to treatment with phorbol esters, with both EAAC1 redistribution to the plasma membrane and the disorganization of actin stress fibers (Fig. 6). Actin presents discrete areas of co-localization with EAAC1 at the cell periphery, a pattern that is detectable both in phorbol-untreated round cells and, in particular, in phorbol-treated flattened cells. On the contrary, in all cells α-adducin appears homogeneously expressed and only partially co-

localized with actin, indicating that only a fraction of the protein is engaged in F-actin binding, as expected from its role of a modulator of microfilament organization [20]. However, in agreement with those literature data[20], actin/adducin co-localization is particularly evident at the cell periphery and at the focal adhesions.

Studies performed in erythrocytes [41], brain [34, 35], and platelets [31, 42] indicated that phosphorylation of α-adducin by PKC inhibits its actin binding activity. Consistently, in phorbol-treated cells no co-localization of actin and α-adducin is detectable. Loss of interaction with adducin may thus contribute to the PKC-mediated changes in actin organization, observed in many cell models and demonstrated here for C6 glioma cells (Fig. 6).

A partial co-localization of α-adducin and EAAC1 is detectable in a few vesicle-like structures in the perinuclear area of cells expressing high levels of the transporter, thus suggesting the existence of distinct intracellular pools of the carrier protein. Thus, while the interaction between EAAC1 and actin cytoskeleton, previously proposed by our group [18], is confirmed by the results presented here, this relationship does not seem to involve α-adducin and, similarly, the pool of α-adducin that is co-localized with EAAC1 appears unrelated to F-actin. Consistently, EAAC1/α-adducin interaction is not lost upon treatment with phorbol esters, despite the marked re-organization of actin cytoskeleton exhibited by phorbol-responsive cells. Rather, the redistribution of EAAC1 carriers caused by the phorbol allows a clear cut visualization of two distinct pools of EAAC1 carriers: one on the membrane, in a close spatial relationship with actin, the other in the perinuclear area, organized in vesicular formations, some of which are also α-adducin-positive. The evidence of a perinuclear co-localization of the two proteins suggest the existence of a newly synthesized pool of EAAC1 carrier which may request an interaction with α-adducin to reach other sub cellular compartments.

Also the co-immunoprecipitation pattern of EAAC1 and α-adducin does not change substantially after PDBu treatment, confirming that the interaction between the two proteins is PKC-independent and does not involve the pool of EAAC1 transporters that are targeted to the plasma membrane when the kinase is activated. Moreover, it is unmodified also after the disorganization of F-actin caused by latrunculin A, confirming that EAAC1/α-adducin interaction is independent from F-actin.

Under all the conditions tested, the bands of EAAC1 co-immunoprecipitated with adducin, although consistently detected, are faint. To test further the interaction, we adopted therefore a condition associated with a significant induction of EAAC1, consisting in the prolonged treatment of C6 cells with ATRA [27]. In ATRA-treated cells, the amount of α-adducin co-immunoprecipitated with EAAC1 markedly increases. Moreover, we demonstrated that a HA-tagged adducin also co-immunoprecipitates with EAAC1 and that the extent of the co-immunoprecipitation seems proportional to EAAC1 expression level. While these results support the specificity of the interaction between the two proteins, they also suggest that the abundance of EAAC1 is the limiting factor in the interaction.

Further experiments will be needed to characterize the vesicle compartments in which adducin and EAAC1 co-localize and interact as well as to verify this interaction in models of differentiated cells of neural origin. However, high expression of α-adducin was described in brain regions with high synapse densities, such as hippocampus, corpus striatum, cerebral cortex and cerebellum. In particular, although it was also detected in processes of glial cells, α-adducin is localized in distinct sub-cellular neuronal structures of CA1 and CA3 regions, such as distinct subsets of dendrites and dendritic spines, while the majority of synaptic structures are adducin-negative [43]. Moreover, in dendritic spines, a role for the protein in the cytoskeletal remodelling that is associated with synaptic stimulation and plasticity has been

proposed [35, 44] and substantiated in studies with β-adducin knock-out mice [45]. Interestingly, in the same subcellular compartments a significant expression of EAAC1 has been also repeatedly described [15, 46-48], raising the possibility that the interaction between EAAC1 and adducin has a significant physiological relevance.

ACKNOWLEDGMENTS

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References

[1] Y. Kanai, M.A. Hediger, The glutamate/neutral amino acid transporter family SLC1: molecular, physiological and pharmacological aspects, Pflugers Arch 447 (2004) 469-479.

[2] A. Nieoullon, B. Canolle, F. Masmejean, B. Guillet, P. Pisano, S. Lortet, The neuronal excitatory amino acid transporter EAAC1/EAAT3: does it represent a major actor at the brain excitatory synapse?, J Neurochem 98 (2006) 1007-1018.

[3] C. Zoia, T. Cogliati, E. Tagliabue, G. Cavaletti, G. Sala, G. Galimberti, I. Rivolta, V. Rossi, L. Frattola, C. Ferrarese, Glutamate transporters in platelets: EAAT1 decrease in aging and in Alzheimer's disease, Neurobiol Aging 25 (2004) 149-157.

[4] K. Aoyama, S.W. Suh, A.M. Hamby, J. Liu, W.Y. Chan, Y. Chen, R.A. Swanson, Neuronal glutathione deficiency and age-dependent neurodegeneration in the EAAC1 deficient mouse, Nat Neurosci 9 (2006) 119-126.

[5] S. Kiryu-Seo, K. Gamo, T. Tachibana, K. Tanaka, H. Kiyama, Unique anti-apoptotic activity of EAAC1 in injured motor neurons, EMBO J 25 (2006) 3411-3421.

[6] K. Aoyama, N. Matsumura, M. Watabe, T. Nakaki, Oxidative stress on EAAC1 is involved in MPTP-induced glutathione depletion and motor dysfunction, Eur J Neurosci 27 (2008) 20-30.

[7] B. Voutsinos-Porche, E. Koning, Y. Clement, H. Kaplan, A. Ferrandon, J. Motte, A. Nehlig, EAAC1 glutamate transporter expression in the rat lithium-pilocarpine model of temporal lobe epilepsy, J Cereb Blood Flow Metab 26 (2006) 1419-1430.

[8] M. Gottlieb, M. Domercq, C. Matute, Altered expression of the glutamate transporter EAAC1 in neurons and immature oligodendrocytes after transient forebrain ischemia, J Cereb Blood Flow Metab 20 (2000) 678-687.

[9] I. Huerta, R.E. McCullumsmith, V. Haroutunian, J.M. Gimenez-Amaya, J.H. Meador-Woodruff, Expression of excitatory amino acid transporter interacting protein transcripts in the thalamus in schizophrenia, Synapse 59 (2006) 394-402.

[10] P.D. Arnold, T. Sicard, E. Burroughs, M.A. Richter, J.L. Kennedy, Glutamate transporter gene SLC1A1 associated with obsessive-compulsive disorder, Arch Gen Psychiatry 63 (2006) 769-776.

[11] T.P. Palos, B. Ramachandran, R. Boado, B.D. Howard, Rat C6 and human astrocytic tumor cells express a neuronal type of glutamate transporter, Brain Res Mol Brain Res 37 (1996) 297-303.

[12] K. Ma, S. Zheng, Z. Zuo, The transcription factor regulatory factor X1 increases the expression of neuronal glutamate transporter type 3, J Biol Chem 281 (2006) 21250-21255.

[13] K.M. Fournier, M.I. Gonzalez, M.B. Robinson, Rapid trafficking of the neuronal glutamate transporter, EAAC1: evidence for distinct trafficking pathways differentially regulated by protein kinase C and platelet-derived growth factor, J Biol Chem 279 (2004) 34505-34513.

[14] K.M. Fournier, M.B. Robinson, A dominant-negative variant of SNAP-23 decreases the cell surface expression of the neuronal glutamate transporter EAAC1 by slowing constitutive delivery, Neurochem Int 48 (2006) 596-603.

[15] F. Conti, S. DeBiasi, A. Minelli, J.D. Rothstein, M. Melone, EAAC1, a high-affinity glutamate tranporter, is localized to astrocytes and gabaergic neurons besides pyramidal cells in the rat cerebral cortex, Cereb Cortex 8 (1998) 108-116.

[16] W. Yang, M.S. Kilberg, Biosynthesis, intracellular targeting, and degradation of the EAAC1 glutamate/aspartate transporter in C6 glioma cells, J Biol Chem 277 (2002) 38350-38357.

[17] A.L. Sheldon, M.I. Gonzalez, M.B. Robinson, A carboxyl-terminal determinant of the neuronal glutamate transporter, EAAC1, is required for platelet-derived growth factor-dependent trafficking, J Biol Chem 281 (2006) 4876-4886.

[18] M.G. Bianchi, B.M. Rotoli, V. Dall'Asta, G.C. Gazzola, R. Gatti, O. Bussolati, PKC-dependent stimulation of EAAT3 glutamate transporter does not require the integrity of actin cytoskeleton, Neurochem Int 48 (2006) 341-349.

[19] R.D. O'Shea, C.L. Lau, M.C. Farso, S. Diwakarla, C.J. Zagami, B.B. Svendsen, S.J. Feeney, J.K. Callaway, N.M. Jones, D.V. Pow, N.C. Danbolt, B. Jarrott, P.M. Beart, Effects of lipopolysaccharide on glial phenotype and activity of glutamate transporters: Evidence for delayed up-regulation and redistribution of GLT-1, Neurochem Int 48 (2006) 604-610.

[20] Y. Matsuoka, X. Li, V. Bennett, Adducin: structure, function and regulation, Cell Mol Life Sci 57 (2000) 884-895.

[21] R. Boito, M. Menniti, R. Amato, C. Palmieri, C. Marinaro, R. Iuliano, G. Tripodi, D. Cusi, G. Fuiano, N. Perrotti, RFX-1, a putative alpha Adducin interacting protein in a human kidney library, FEBS Lett 579 (2005) 6439-6443.

[22] P.G. Laustsen, W.S. Lane, V. Bennett, G.E. Lienhard, Association of protein kinase C(lambda) with adducin in 3T3-L1 adipocytes, Biochim Biophys Acta 1539 (2001) 163-172.

[23] M. Ferrandi, S. Salardi, G. Tripodi, P. Barassi, R. Rivera, P. Manunta, R. Goldshleger, P. Ferrari, G. Bianchi, S.J. Karlish, Evidence for an interaction between adducin and Na(+)-K(+)-ATPase: relation to genetic hypertension, Am J Physiol 277 (1999) H1338-1349.

[24] R. Efendiev, R.T. Krmar, G. Ogimoto, J. Zwiller, G. Tripodi, A.I. Katz, G. Bianchi, C.H. Pedemonte, A.M. Bertorello, Hypertension-linked mutation in the adducin alpha-subunit leads to higher AP2-mu2 phosphorylation and impaired Na+,K+-ATPase trafficking in response to GPCR signals and intracellular sodium, Circ Res 95 (2004) 1100-1108.

[25] S.H. Rozen S, Primer3 on the WWW for general users and for biologist programmers Methods Mol Biol 132 (2000) 365-386.

[26] S.A. Bustin, Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays, J Mol Endocrinol 25 (2000) 169-193.

[27] M.G. Bianchi, G.C. Gazzola, L. Tognazzi, O. Bussolati, C6 glioma cells differentiated by retinoic acid overexpress the glutamate transporter excitatory amino acid carrier 1 (EAAC1), Neuroscience 151 (2008) 1042-1052.

[28] K. Ohira, K.J. Homma, H. Hirai, S. Nakamura, M. Hayashi, TrkB-T1 regulates the RhoA signaling and actin cytoskeleton in glioma cells, Biochem Biophys Res Commun 342 (2006) 867-874.

[29] M. Casado, A. Bendahan, F. Zafra, N.C. Danbolt, C. Aragon, C. Gimenez, B.I. Kanner, Phosphorylation and modulation of brain glutamate transporters by protein kinase C, J Biol Chem 268 (1993) 27313-27317.

[30] F. Tatin, C. Varon, E. Genot, V. Moreau, A signalling cascade involving PKC, Src and Cdc42 regulates podosome assembly in cultured endothelial cells in response to phorbol ester, J Cell Sci 119 (2006) 769-781.

[31] D.M. Gilligan, R. Sarid, J. Weese, Adducin in platelets: activation-induced phosphorylation by PKC and proteolysis by calpain, Blood 99 (2002) 2418-2426.

[32] K.E. Davis, D.J. Straff, E.A. Weinstein, P.G. Bannerman, D.M. Correale, J.D. Rothstein, M.B. Robinson, Multiple signaling pathways regulate cell surface expression and activity of the excitatory amino acid carrier 1 subtype of Glu transporter in C6 glioma, J Neurosci 18 (1998) 2475-2485.

[33] M.I. Gonzalez, P.G. Bannerman, M.B. Robinson, Phorbol myristate acetate-dependent interaction of protein kinase Calpha and the neuronal glutamate transporter EAAC1, J Neurosci 23 (2003) 5589-5593.

[34] V. Bennett, K. Gardner, J.P. Steiner, Brain adducin: a protein kinase C substrate that may mediate site-directed assembly at the spectrin-actin junction, J Biol Chem 263 (1988) 5860-5869.

[35] Y. Matsuoka, X. Li, V. Bennett, Adducin is an in vivo substrate for protein kinase C: phosphorylation in the MARCKS-related domain inhibits activity in promoting spectrin-actin complexes and occurs in many cells, including dendritic spines of neurons, J Cell Biol 142 (1998) 485-497.

[36] J.R. Peterson, T.J. Mitchison, Small molecules, big impact: a history of chemical inhibitors and the cytoskeleton, Chem Biol 9 (2002) 1275-1285.

[37] M. Jackson, W. Song, M.Y. Liu, L. Jin, M. Dykes-Hoberg, C.I. Lin, W.J. Bowers, H.J. Federoff, P.C. Sternweis, J.D. Rothstein, Modulation of the neuronal glutamate transporter EAAT4 by two interacting proteins, Nature 410 (2001) 89-93.

[38] C.I. Lin, I. Orlov, A.M. Ruggiero, M. Dykes-Hoberg, A. Lee, M. Jackson, J.D. Rothstein, Modulation of the neuronal glutamate transporter EAAC1 by the interacting protein GTRAP3-18, Nature 410 (2001) 84-88.

[39] H. Marie, D. Billups, F.K. Bedford, A. Dumoulin, R.K. Goyal, G.D. Longmore, S.J. Moss, D. Attwell, The amino terminus of the glial glutamate transporter GLT-1 interacts with the LIM protein Ajuba, Mol Cell Neurosci 19 (2002) 152-164.

[40] H. Marie, S.J. Pratt, M. Betson, H. Epple, J.T. Kittler, L. Meek, S.J. Moss, S. Troyanovsky, D. Attwell, G.D. Longmore, V.M. Braga, The LIM protein Ajuba is recruited to cadherin-dependent cell junctions through an association with alpha-catenin, J Biol Chem 278 (2003) 1220-1228.

[41] E. Ling, K. Gardner, V. Bennett, Protein kinase C phosphorylates a recently identified membrane skeleton-associated calmodulin-binding protein in human erythrocytes, J Biol Chem 261 (1986) 13875-13878.

[42] K.L. Barkalow, J.E. Italiano, Jr., D.E. Chou, Y. Matsuoka, V. Bennett, J.H. Hartwig, Alpha-adducin dissociates from F-actin and spectrin during platelet activation, J Cell Biol 161 (2003) 557-570.

[43] B. Seidel, W. Zuschratter, H. Wex, C.C. Garner, E.D. Gundelfinger, Spatial and sub-cellular localization of the membrane cytoskeleton-associated protein alpha-adducin in the rat brain, Brain Res 700 (1995) 13-24.

[44] F. Angenstein, K. Buchner, S. Staak, Age-dependent differences in glutamate-induced phosphorylation systems in rat hippocampal slices, Hippocampus 9 (1999) 173-185.

[45] R.L. Rabenstein, N.A. Addy, B.J. Caldarone, Y. Asaka, L.M. Gruenbaum, L.L. Peters, D.M. Gilligan, R.M. Fitzsimonds, M.R. Picciotto, Impaired synaptic plasticity and learning in mice lacking beta-adducin, an actin-regulating protein, J Neurosci 25 (2005) 2138-2145.

[46] C. Cheng, G. Glover, G. Banker, S.G. Amara, A novel sorting motif in the glutamate transporter excitatory amino acid transporter 3 directs its targeting in Madin-Darby canine kidney cells and hippocampal neurons, J Neurosci 22 (2002) 10643-10652.

[47] P. Meera, P.D. Dodson, M.H. Karakossian, T.S. Otis, Expression of GFP-tagged neuronal glutamate transporters in cerebellar Purkinje neurons, Neuropharmacology 49 (2005) 883-889.

[48] C. Genoud, C. Quairiaux, P. Steiner, H. Hirling, E. Welker, G.W. Knott, Plasticity of astrocytic coverage and glutamate transporter expression in adult mouse cortex, PLoS Biol 4 (2006) e343.

Figure Legends

Fig. 1. Expression of EAAC1 in C6 glioma cells. C6 cells, seeded on four-chamber coverslides and incubated in normal growth medium, were fixed and stained with anti-EAAC1 antibody (Panel A, *red*) and phalloidin (Panel B, *green*). A single confocal section of a representative field is shown. High levels of EAAC1 expression were detected in round bipolar cells (Panel A, arrowheads) while spread cells had low to intermediate levels of expression. In both cell types, EAAC1 expression was mostly detected in vesicles particularly abundant in the perinuclear region (Panel A, arrows). Panel C shows the composite image. White rectangles delimit areas, magnified in the insets, that show clusters of co-localization in *yellow*. See Experimental Procedures for details. Bar = 20 μm.

Fig. 2. Expression of α-adducin in C6 glioma cells. Cells were fixed and stained with phalloidin (Panel A) and anti-α-adducin antibody (Panel B). A single confocal section of a representative field is shown. In the acquired images, the intensity of adducin signal is rendered in a *green* scale, while the actin signal is rendered in *red*. Panel C shows the composite image where the co-localization spots (*yellow*) are detectable. Areas of actin/adducin co-localization are detected at the focal adhesions (arrows) and in the cortical regions of some cells (arrowheads). Bar = 20 μm.

Fig.3. Co-localization of EAAC1, α-adducin and actin in C6 glioma cells. Cells were treated as described in Fig. 3. (Panel A, *red*), anti-adducin antibody (Panel B, *green*), and phalloidin (Panel C, *blue*). A single confocal section of a representative field are shown. The composite image (Panel D) and the magnified images (insets) show areas of co-localization. In a round bipolar cell, an appreciable co-localization of the three probes (*white*) is detectable at the end

of one of the cells processes (*arrowhead*), while in the other process only EAAC1 and actin co-localize (*magenta*, *arrows*). In spread cells, while actin and adducin are partially co-localized at the cell periphery (*cyan, open arrowhead*), EAAC1 transporters are distributed mainly around cell nuclei where they appear partially co-localized with α-adducin (*yellow*, *open arrow*). Bar = 20 μm.

Fig. 4. Co-localization of EAAC1, α-adducin and actin in C6 glioma cells. Cells were fixed and stained as in Figure 3. Anti-EAAC1 antibody (Panel A, *red*), anti-adducin antibody (Panel B, *green*), phalloidin (Panel C, *blue*), composite image (Panel D). A single confocal section of a selected field is shown. Areas of co-localization of EAAC1 and α-adducin (*yellow*) are highlighted by *arrows*. Bar = 10 μm.

Fig. 5. Co-localization of EAAC1, α-adducin and actin in C6 glioma cells. Cells were fixed and stained as in Figure 3. Anti-EAAC1 antibody (Panel A, *red*), anti-adducin antibody (Panel B, *green*), phalloidin (Panel C, *blue*), composite image (Panel D). A single confocal section of a selected field is shown. An area of co-localization of EAAC1 and α-adducin (*yellow*) is highlighted by the *arrow*. Bar = 10 μm.

Fig. 6. The expression of EAAC1, α-adducin and actin in C6 glioma cells: effect of phorbol esters. Before fixation, cells were treated for 15 min with 150 nM PDBu. Cells were then stained with anti-EAAT3 antibody (Panel A, *red*), anti-adducin antibody (Panel B, *green*), and phalloidin (Panel C, *blue*). A single confocal section of a representative field is shown. The composite image (Panel D) and the magnified images (insets) show areas of co-localization. PDBu treatment causes an increase of EAAC1 transporters on the plasma membrane (Panel

A, *arrowheads*) and the appearance of podosome-like structures in membrane ruffles (Panel C, *open arrowheads*). Partial EAAC1/adducin (*yellow, open arrows*) and EAAC1/actin (*magenta, arrows*) co-localizations are evident in some cells (Panel D). Bar = 20 μm.

Fig. 7. Anti-EAAC1 antibodies co-immunoprecipitate α-adducin in C6 glioma cells. Panels A and B. C6 cells were incubated for 15 min in the absence or in the presence of 150 nM PDBu, as indicated. Panel A. Cell lysates were immunoprecipitated with an anti-EAAC1 antibody (IP:EAAC1) or no antibody (Beads) and precipitates were probed for immunoreactivity to adducin. On the right, Western Blot of α-adducin in C6 lysates. Panel B. Lysates of PDBu-treated or control cells were probed for EAAC1 immunoreactivity before (Lysate) or after (Surnatant) immunoprecipitation with anti-EAAC1 antibody. Panels C and D. C6 cells were incubated for 15 min in the absence or in the presence of 0.6 μM latrunculin, as indicated. Cell lysates were treated as described in Panels A and B. The experiments was repeated twice with comparable results.

Fig.8. ATRA-induced EAAC1 overexpression increases adducin-EAAC1 co-immunoprecipitation. C6 cells were incubated for 4d in the absence or in the presence of 10 μM ATRA, as indicated. Panel A. IP, precipitates with anti-EAAC1 antibody (IP:EAAC1) or no antibody (Beads). Precipitates were probed for immunoreactivity to adducin. On the right, Western Blot of α-adducin in C6 lysates. Panel B. Lysates of ATRA-treated or control cells were probed for EAAC1 immunoreactivity before (Lysate) or after (Surnatant) immunoprecipitation with anti-EAAC1 antiserum. The experiment was repeated twice with comparable results. Note that EAAC1 expression in control cells seems lower in this

experiment due to the short exposure time adopted so as to avoid EAAC1 signal saturation in ATRA-treated cells.

Fig. 9. Adducin-EAAC1 co-immunoprecipitation in C6 clones transfected with HA-adducin. Panel A. The levels of expression of Adducin, EAAC1 and HA-Adducin were determined with Western Blot in the indicated clones of C6 cells transfected with HA-Adducin (see Methods). 30 μg of protein lysates were used in each lane. The experiment was repeated twice with comparable results. Panel B. Uptake of D-Asp was determined, as described in Materials and Methods, in parental C6 cells and in clones 11 and 21, as indicated. Data are means of five independent determinations ± SD. Panel C. The expression of Slc1a1 was determined, as described in Materials and Methods, in parental C6 cells and in clones 11 and 21, as indicated. Data are means of four determinations obtained in two experiments ± SD. Panel D. IP, precipitates with anti-HA-adducin antibody (IP:HA) or no antibody (Beads). Precipitates were probed for immunoreactivity to EAAC1 or HA-adducin. On the right, Western Blot of α-adducin in C6 lysates. The experiment was repeated twice with comparable results. Panels B and C, ** $p < 0.01$ vs. parental C6 cells.

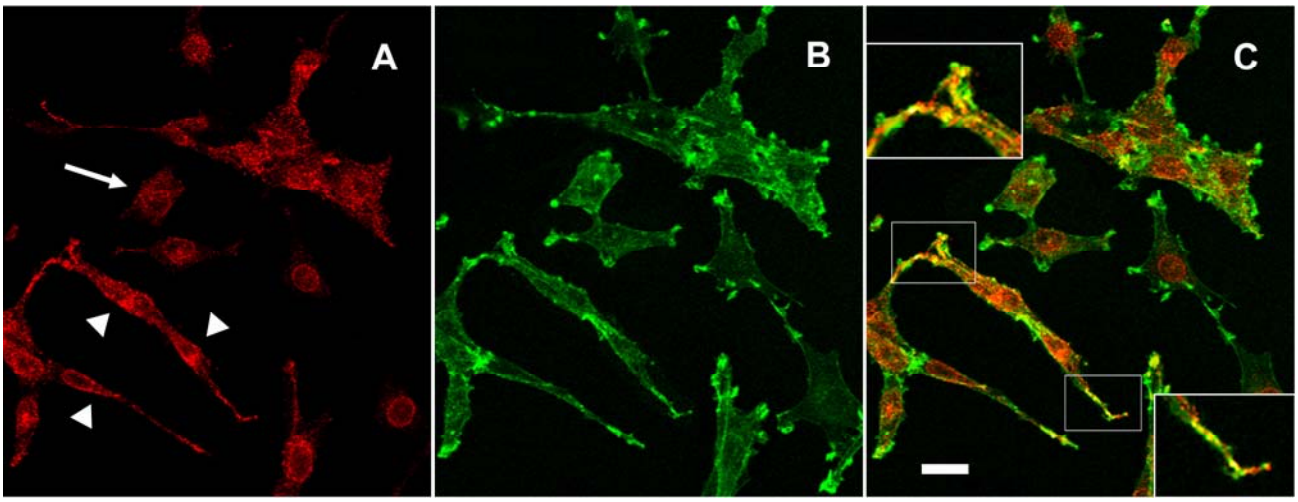

Figure 1

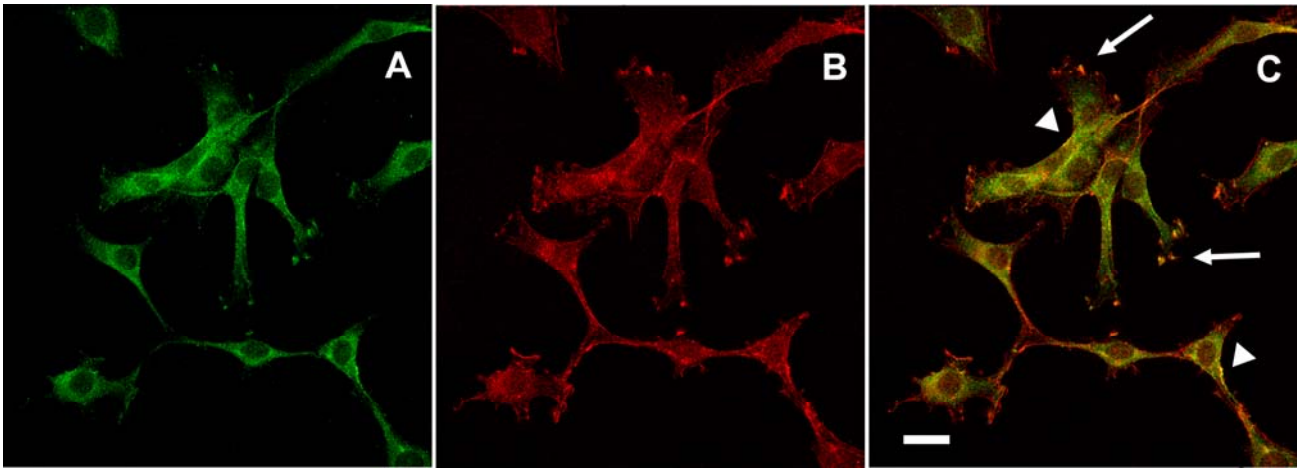

Figure 2

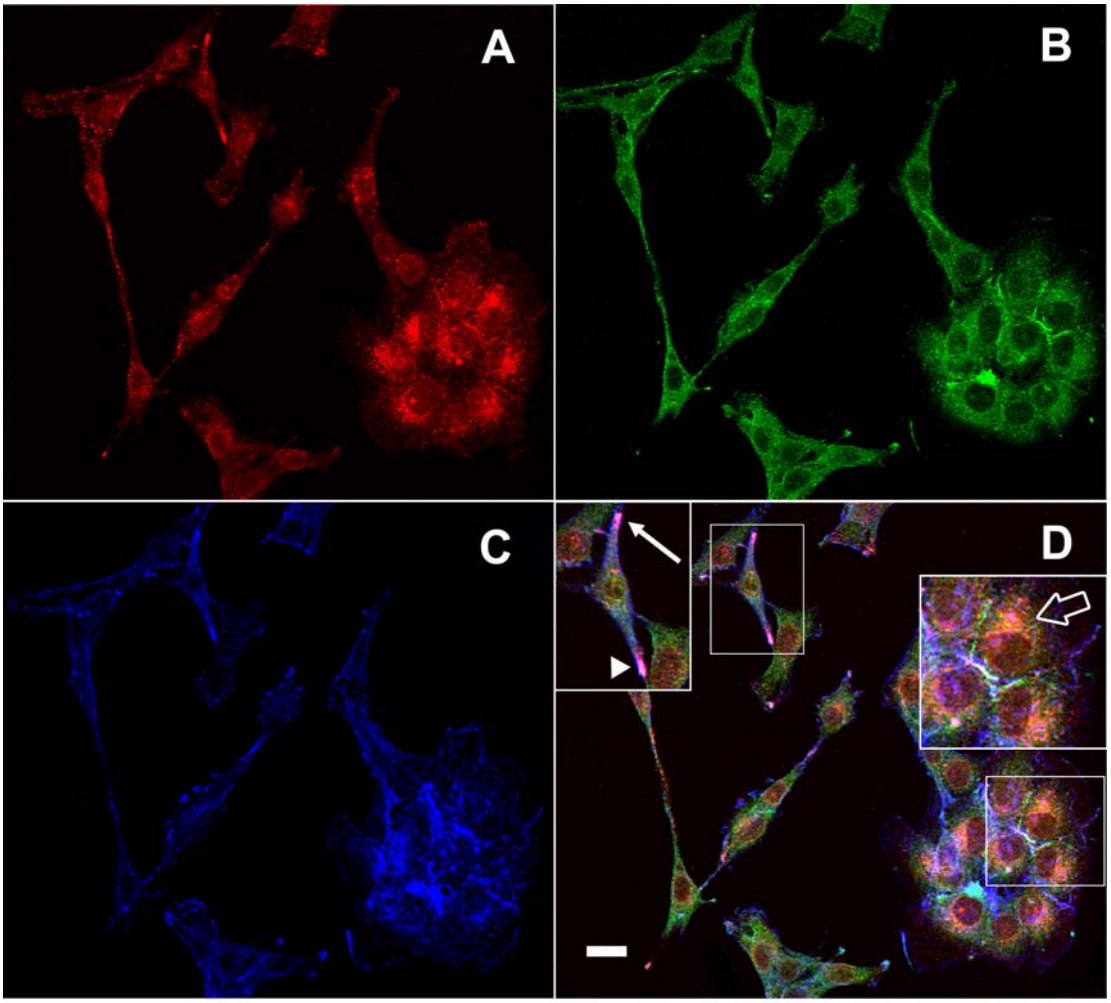

Figure 3

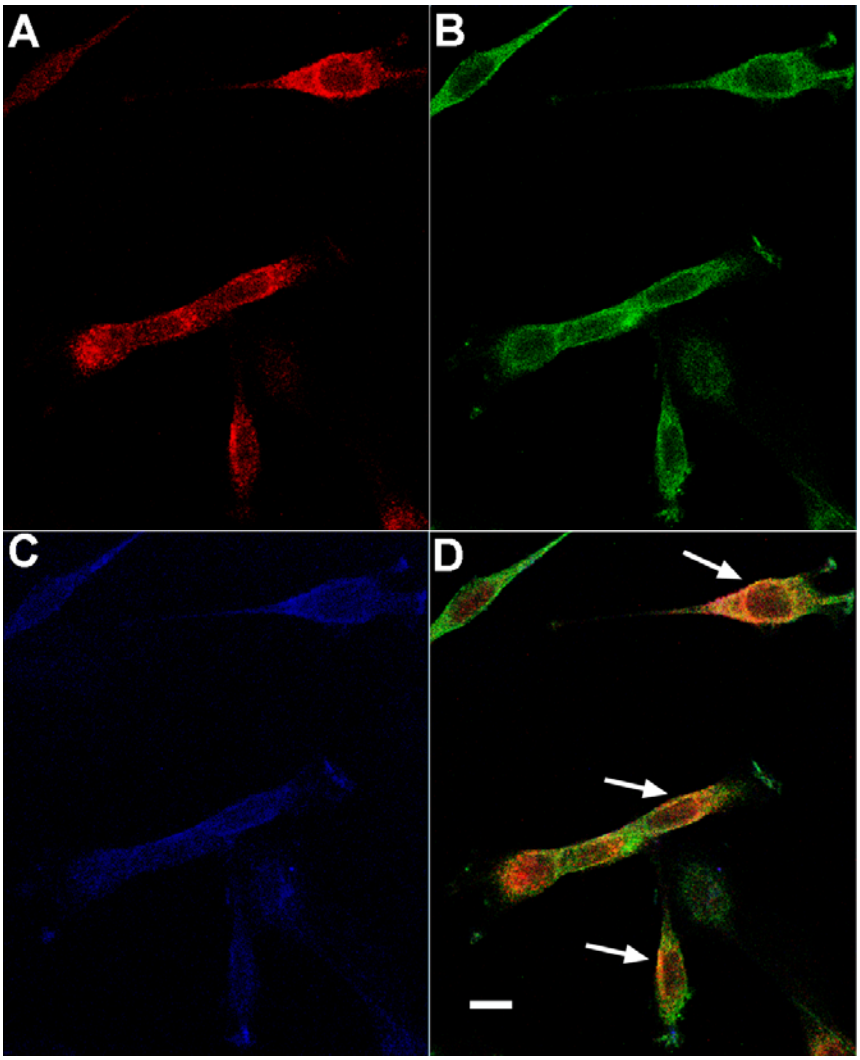

Figure4

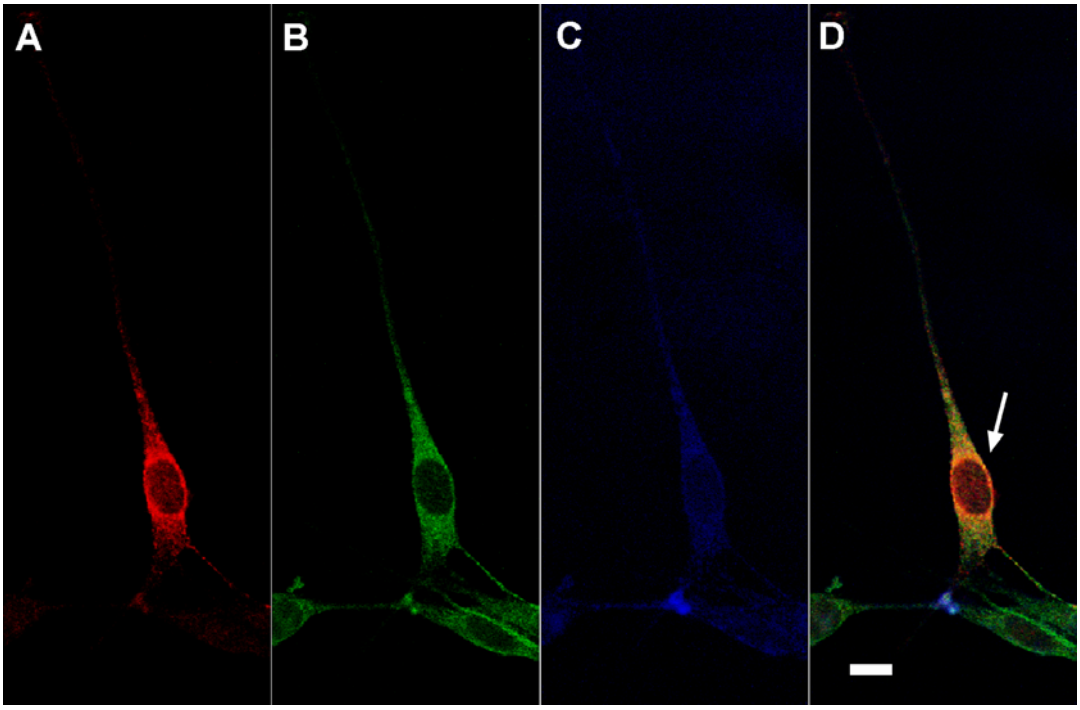

Figure 5

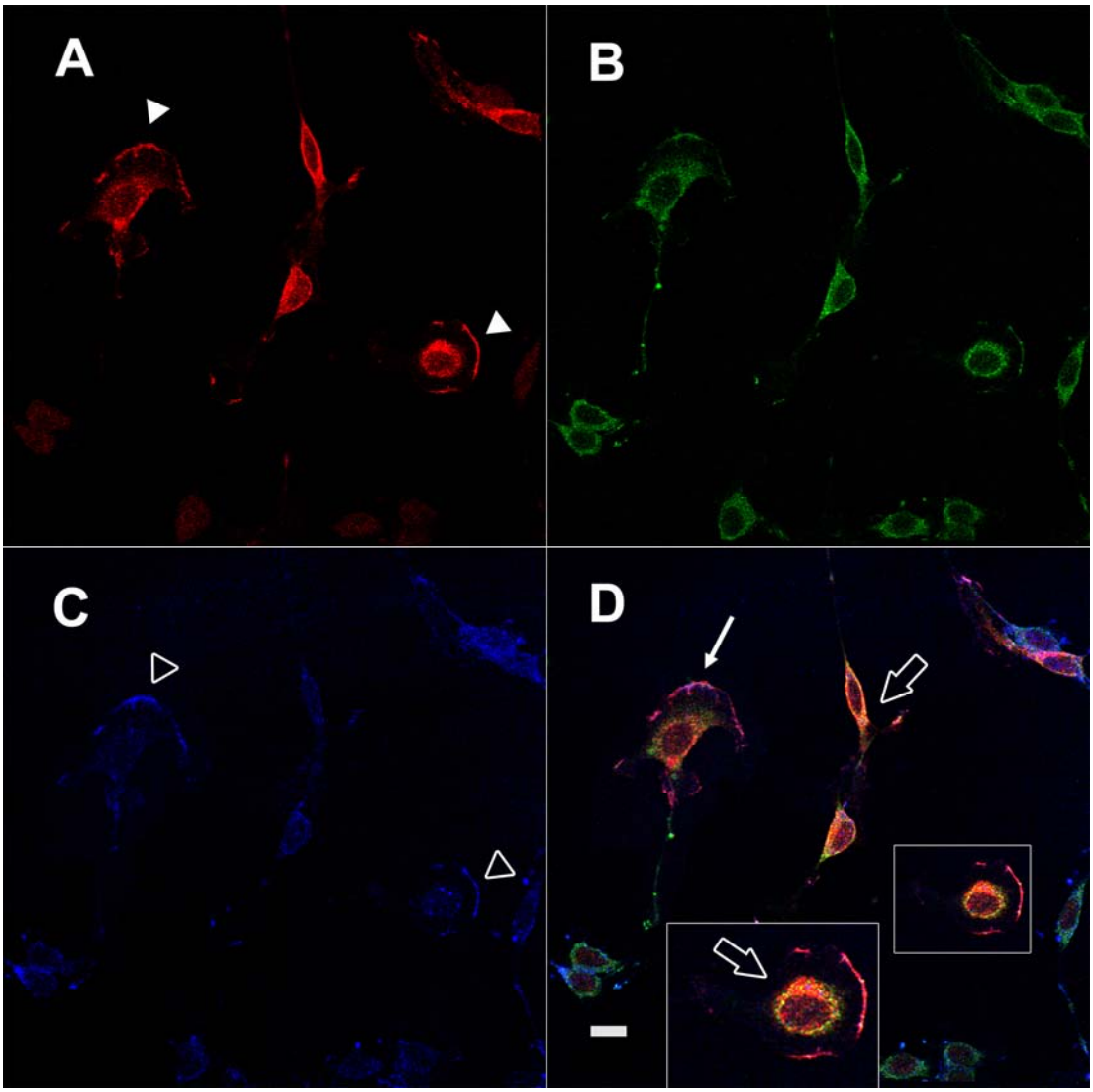

Figure 6

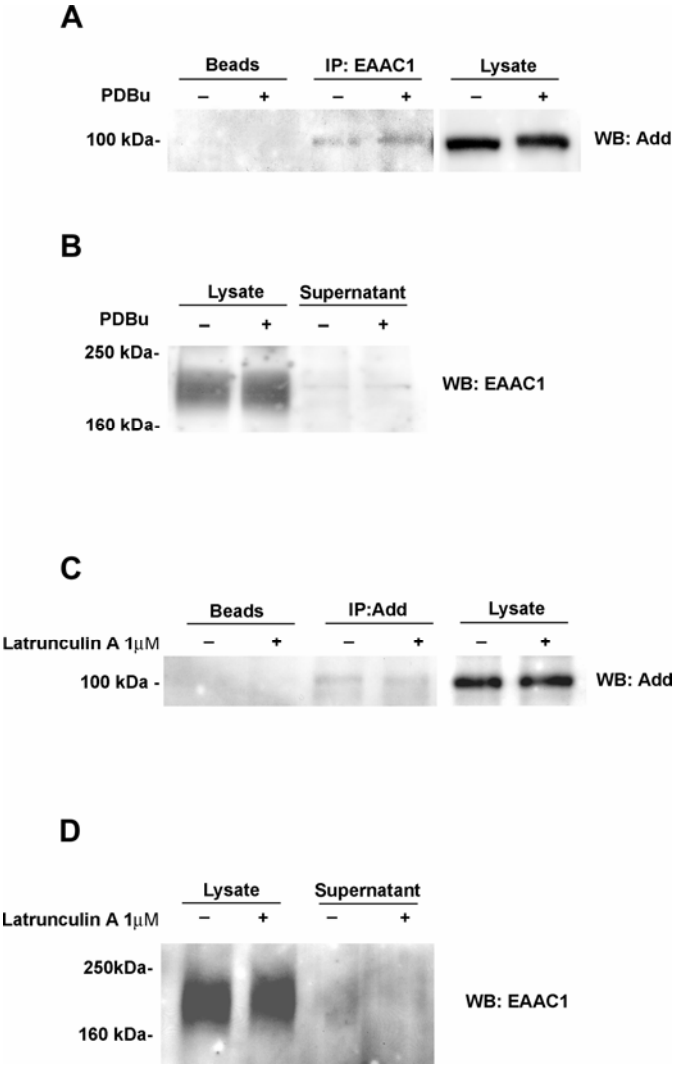
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Figure 7

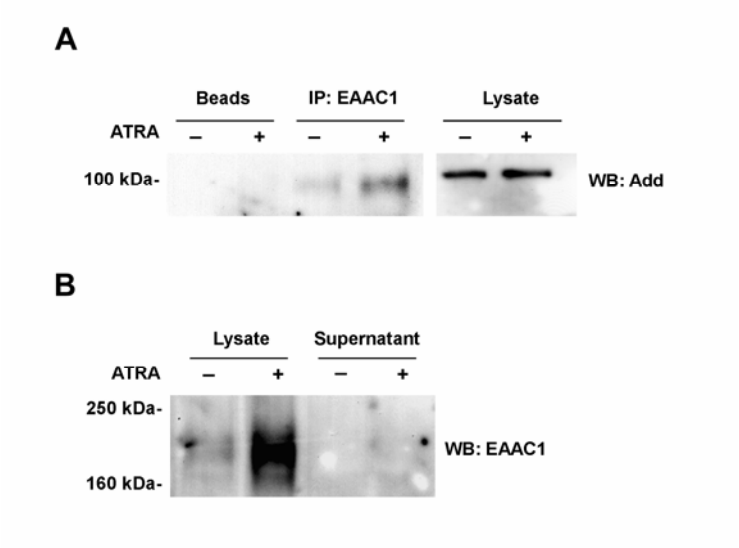
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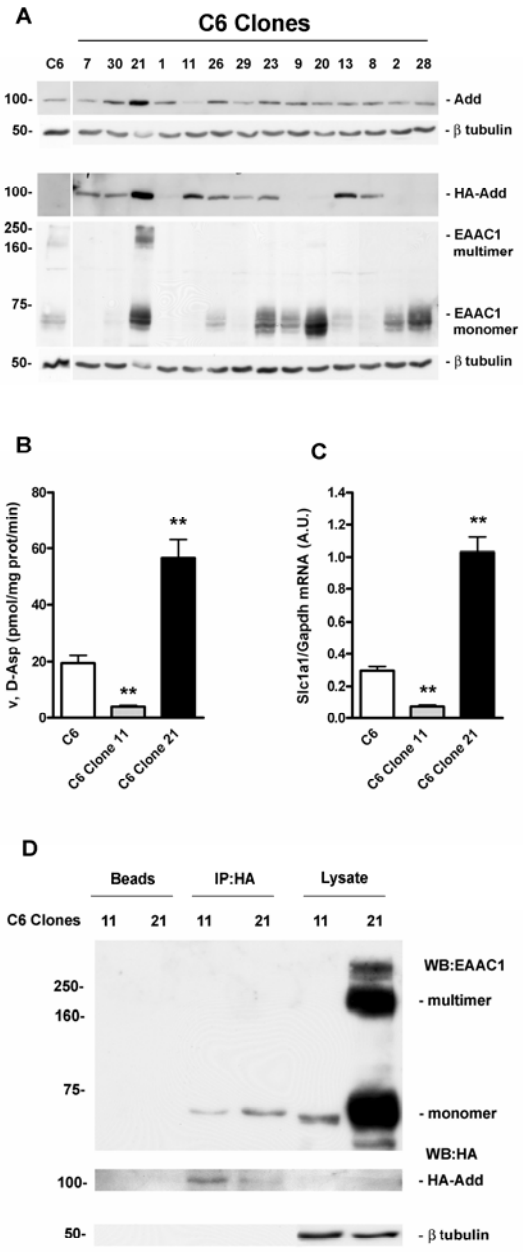
Figure 8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- EAAC1 multimer
75-
- EAAC1 monomer
50-
- β tubulin
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Figure 9

The results presented above point to the existence of a pool of EAAC1 carriers interacting with adducin in specific subcellular compartments, to be identified, mainly located in the perinuclear region. Neither co-immunoprecipitation nor co-localization were affected by PKC-activation, consistently with the existence of at least two intracellular pools of EAAC1 carriers, one related to actin cytoskeleton (possibly though an interaction that involves also adducin) and the other, unrelated to the actin cytoskeleton and sensitive to PKC activation. Although the co-immunoprecipitation bands were very faint, we rely on three evidences to support the existence of the interaction: a) the same pattern was observed with anti-HA antibodies in C6 cells transfected with HA-adducin, yielding a remarkable evidence of specificity; b) using two clones of C6 cells endowed with very different transport activities, we observed co-immunoprecipitation bands of different intensities, roughly proportional to aspartate influx and Slc1a1 expression; c) a condition associated with EAAC1-overexpression, i.e. the chronic treatment with retinoic acid, produced more evident co-immunoprecipitation bands.

Altogether, these results indicated that adducin could be a novel molecular partner of EAAT3/EAAC1 and, given the evidences pointing to the presence of the two protein in dendritic spines of hippocampal neurons, should prompt further investigations on the interaction adducin-EAAT3/EAAC1 in situ or in other in vitro model of differentiation. While the results obtained suggested that EAAC1 protein may be the limiting partner for the interaction with adducin, the last approach adopted yielded an experimental device able to increase EAAC1 expression, providing hints on the regulation of EAAC1 at the gene level, a field quite incompletely explored so far.

The rationale for this approach was provided by the finding of a stem cell component of C6 cultures able to go thorough distnct differentative pathways (Lopez-Barahona et al., 1993; Zheng et al., 2007). Thus we decided to test if any differentiating condition may affect EAAC1 expression and, hence, the EAAC1-adducin interaction. Actually, we found that ATRA was able to stimulate significantly aspartate transport and EAAC1 expression after some unfruitful attempts with thyroid hormones, inhibitors of HDAC, and cAMP analogues. Thus, ATRA effects on EAAC1 expression representing the first example of the induction of the EAAT3 carrier by a differentiating agent and, hence, were worthy to be characterized further.

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C6 GLIOMA CELLS DIFFERENTIATED BY RETINOIC ACID OVEREXPRESS THE GLUTAMATE TRANSPORTER EAAC1.

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Abbreviations

ATRA, all-*trans*-retinoic acid; DHK, dihydrokainate; DL-TBOA, DL-threo-b-Benzyloxyaspartic acid; DMEM, Dulbecco's modified Eagle Medium; EAAC1, Excitatory Amino Acid Carrier 1; EAAT, Excitatory Amino Acid Transporter; FBS, Fetal Bovine Serum; GFAP, Glial Fibrillary Acidic Protein; L-CSS, L-cysteinsulfinic acid; PDBu, Phorbol 12,13-dibutyrate; PLP, proteolipid protein; TUBB3, Class III β-tubulin.

Abstract

The transport of excitatory anionic amino acids in central nervous system is performed by a family of high affinity, sodium dependent carriers. One of these transporters, EAAC1, is expressed mostly in neurons and is mainly regulated by mechanisms that modify carrier abundance on the plasma membrane. Much less is known on EAAC1 regulation at the level of gene expression. Here we report that in C6 rat glioma cells, a line recently described to contain neural stem-like cells, EAAC1 is markedly induced by all *trans*-retinoic acid (ATRA), a well known differentiating agent. Consistently, ATRA stimulates anionic amino acid transport, with the maximal effect observed at concentrations $\geq$ 1 μM. After 4 days of treatment with 10 μM ATRA, the transport V_{max} is five-fold enhanced, Slc1a1 mRNA is increased by 400% compared to control, EAAC1 carrier is 6-fold overexpressed and the C6 culture is greatly enriched of cells with bipolar morphology strongly positive for EAAC1 immunoreactivity. Compared to untreated cells, ATRA-treated C6 cells express less Slc1a3 mRNA, for the carrier GLAST, but significantly higher levels of Slc1a2 mRNA, for the transporter GLT-1, although no expression of this protein is detected with Western Blot in both untreated and ATRA-treated cells. Consistently, the inhibition pattern of aspartate transport and its stimulation by phorbol esters are indicative of a transport process due to EAAC1 operation. Under the conditions adopted, ATRA treatment causes the induction of ProteoLipid Protein, an oligodendrocytic marker. These results indicate that, in C6 cells, ATRA stimulates the expression of EAAC1 and constitute the first demonstration of the induction of this transporter by a differentiating agent.

Keywords:

Neural stem cells; excitatory amino acids; excitotoxicity; oligodendrocytes; differentiation; EAAT3

Rat glioma C6 cells represent one of the most widely used models for the study of glutamate transport through the “neuronal-type” EAAC1 transporter, the product of Slc1a1 gene. This cell line derives from a glioma induced with nitrosourea (Benda et al., 1968) and can be driven towards different differentiation pathways (Parker et al., 1980), as demonstrated by the expression of astrocytic (Segovia et al., 1994; Takanaga et al., 2004; Lind et al., 2006), oligodendrocytic (McMorris, 1983; Lopez-Barahona et al., 1993; Gravel et al., 2000; Pflug et al., 2001) as well as neuronal markers (Segovia et al., 1994) depending on the experimental conditions adopted. In agreement with these data, neural stem cells may represent a substantial portion of the cell population in C6 cultures (Kondo et al., 2004; Setoguchi et al., 2004; Zheng et al., 2007).

All-*trans* retinoic acid (ATRA) plays an important role in the development and regeneration of the nervous system (for a recent review see (Maden, 2007)). In particular, ATRA induces the differentiation of embryonic and CD133+ hematopoietic stem cells into neurones, astrocytes, and oligodendrocytes (Dinsmore et al., 1996; Sanchez-Ramos et al., 2001; Jang et al., 2004). In C6 cells ATRA hinders cell proliferation and motility (Fischer et al., 1987; Lopez-Barahona et al., 1993) and promotes the expression of oligodendrocytic markers (Zhu et al., 1992; Lopez-Barahona et al., 1993).

In some cell models, ATRA is also known to cause changes in the expression of glutamate transporters. In particular, treatment with ATRA had been associated to the increased expression of an unidentified sodium-glutamate co-transporter, along with other co-transporters of amino acids, in opossum kidney cells (de Toledo et al., 1997). More recently, the differentiation of neural stem cells into glial cells by a mixture of differentiating agents, including ATRA, has been found to promote the expression of glial-type glutamate transporters and to increase the uptake of anionic amino acids (Vanhoutte et al., 2004). A significant stimulation of sodium-dependent glutamate transport by retinoic acid has been also described in HEK293 human kidney cells, although this effect seems dissociated by

changes in the expression of EAAT3, the human counterpart of EAAC1 which is the predominant transporter in that cell model (Butchbach et al., 2003).

In the light of the increasing evidence for stem-like properties of C6 cells, we have investigated if the ATRA-induced differentiation is associated to changes in anionic amino acid transport in this cell line. Here we show that ATRA increases the expression of Slc1a1, leading to enhanced levels of EAAC1 protein and to a marked stimulation of anionic amino acid influx.

Experimental procedures

2.1 Cells and experimental treatments

The rat central nervous system-derived C6 glioma cells were obtained from American Type Culture Collection (Rockville, MD). Cells were grown at 37°C and 5% CO_2 in Dulbecco's modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 4 mM glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin. Cells were used for less than 10 passages from thawing with no apparent change in morphology or sensitivity to treatments. Images of cells in phase contrast were obtained with a Nikon DS5MC digital camera.

For ATRA treatment, culture medium was substituted with fresh medium supplemented with 0.1% FBS in the absence or in the presence of ATRA 12h after the passage. ATRA, obtained from Sigma, was used at a concentration of 10 μM from a 10 mM stock solution in DMSO.

2.2 Cell growth

Cell growth was assessed with the viability indicator resazurin (O'Brien et al. 2000). Cells were seeded in complete growth medium in 96-well (Costar, Corning Incorporated, Corning, NY, USA), at a density of $10 \cdot 10^3$ cells/well. At the experimental times, cell viability was tested replacing medium with a solution of resazurin (44 μM) in complete medium. After 1h, fluorescence was measured at 572 nm with a fluorimeter (Wallac 1420 Victor2 Multilabel Counter, Perkin Elmer).

2.3. Determination of transport activity

The initial influx of L-[^{3}H]aspartate or of D-[^{3}H]aspartate, both high-affinitysubstrates of excitatory amino acid (EAA) transporters (Kanai and Hediger, 2003), was measured in 96-well multidish plates (Falcon, Becton, Dickinson Biosciences, Franklin Lakes, NJ, USA)

where C6 cells had been seeded at a density of $10 \cdot 10^3$ cells/well. For the experiments, cells were washed twice in a modified EBSS (Earle's Balanced Salt Solution, buffered with 20 mM HEPES-NaOH at pH 7.4) and incubated in the same saline solution supplemented with L-[^{3}H]aspartate or D-[^{3}H]aspartate (1 µM, 2µCi/ml) for 1 min. For phorbol pre-treatment, phorbol 12,13-dibutyrate (PDBu) was added to growth medium 15 min before the transport assay at a concentration of 150 nM from a 1 mM stock solution in ethanol. Inhibitors of anionic amino acid transport were used at the concentrations indicated for each experiment from 100 mM stock solutions in medium. For the measurement of Na^+-independent uptake, a Na^+-free EBSS was used in which sodium chloride and HEPES-NaOH were substituted by equimolar N-methyl-D-glucamine chloride and Tris-HCl, respectively. At the end of the assay, multiwell dishes were washed twice with ice-cold urea (300 mM) and cell monolayers were extracted with 50 µl of absolute ethanol. The extracts were added to 200 µl of scintillation fluid and counted with a Wallac Trilux^2 liquid scintillation spectrometer (Perkin-Elmer, Boston, MA, USA). Cell proteins were determined directly in the well with a modified Lowry procedure and measured with a Wallac Victor^2 Multilabel Counter (Perkin-Elmer).

For the determination of kinetic constants, the uptake of L-Asp was determined in a range of concentrations from 1 µM to 256 µM,. Data were fitted with a non linear regression analysis to the equation

$$v = \frac{V_{max} * [S]}{K_m + [S]} + K_D * [S] \qquad \text{(Eq. 1)}$$

that describes L-Asp transport as the sum of a single saturable component and of a low-affinity, quasi-diffusive component (Gazzola et al., 1981). The analysis was performed with GraphPad Prism4™ software.

2.4 Immunofluorescence

C6 cells, seeded on coverslips at a density of $20 \cdot 10^3$ cells/cm^2, were rinsed in PBS and fixed for 10 min in 3.7% paraformaldehyde in PBS. After two further rinses, cells were permeabilized with 0.1% Triton X-100 in PBS and incubated for 1h in blocking solution (2% of BSA in PBS) supplemented with anti-EAAC1 polyclonal antibody (Alpha Diagnostics Intl. Inc., San Antonio, TX, USA; 1:100) and, after proper washing, with Alexa Fluor 543 anti-rabbit antibody (Invitrogen, Paisley, UK, 1:400) for an additional 1h. After two further rinses, actin was stained with a 20-min incubation in the presence of AlexaFluor 633 phalloidin (Invitrogen, 4U/ml). The coverslips were then mounted and analyzed with a Zeiss® 510 LSM Meta confocal microscope using a multi-track detection system. A 63X (NA 1.4 oil) objective was used. EAAC1 signal is rendered with a scale of reds (from 0 to 256), while actin is rendered in a scale of greens (from 0 to 256). Palettes are reported in Figure 6. The LSM 5® software, Version 3.5, was used to analyze the images so as to obtain the number of positive pixels and the mean signal intensity.

2.5 Western Blot

C6 cells, grown to subconfluence on 10-cm tissue culture plates, were rinsed twice in PBS and incubated in 800 µl of RIPA buffer (10 mM phosphate buffer pH 7.5, 80 mM NaCl, 0.1% Igepal, 1 mM orthovanadate) containing a cocktail of protease inhibitors for 1h at 4°C. After solubilization, cell lysates were collected in eppendorf tubes, passed 10 times into 26G needles, and centrifuged at 14000 g for 10 min. The supernatants were transferred into new tubes, quantification of proteins was performed with Biorad assay

method and aliquots of 15 µg were loaded on an 8% gel for SDS-PAGE. After the electrophoresis, proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories S.r.l., Segrate, Italy). Non-specific binding sites were blocked with an incubation in Tris-buffer saline (TBS; 50 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 10% of blocking reagent (Roche SpA, Monza, Italy) for 2h at room temperature. The blots were then exposed overnight at 4°C to anti-EAAC1 polyclonal antiserum (1:2000), obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), diluted in blocking solution. After washing, the blots were exposed for 1h to horseradish peroxidase-conjugated anti-rabbit IgG (ExactaCruz, Santa Cruz Biotechnology) diluted 1:30000 in blocking solution. For the standardization of the total cell lysate and the intracellular fraction, stripped membranes were exposed to monoclonal anti-rabbit antiserum against GAPDH (Chemicon International Inc., Temecula, CA, USA, 1:4000). The membranes were washed and the immunoreactivity visualized with enhanced chemiluminescence (Amersham Pharmacia Biotech Italia, Milan, Italy).

For the assessment of GLT-1 and GLAST expression, 20 µg of C6 lysates were electrophoresed in parallel with 100 µg of rat brain extract (Santa Cruz Biotechnology) and probed with anti-GLT1 or anti-GLAST antisera (Tocris Cookson, Bristol, UK, both at 1:1000). Cell lysates and the brain extract were boiled for 5 min before PAGE.

2.6. qRT-Polymerase Chain Reaction

Total RNA was isolated with RNeasy Mini Kit® (Qiagen S.p.a., Milan, Italy). After reverse transcription, 25 ng of cDNA from each sample was amplified in a total volume of 25 µl with 2X Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen), along with the following forward and reverse primers (5 pmol each) for the genes: 5’ CGA CTT GCC GTA CCT GGA CT 3’ and 5’ GCC CAC GGG ACT CAA CAC GA 3’ for Slc1a1; 5’ ACA ATA TGC CCA AGC AGG TAG A 3’ and 5’ GAC ACC AAA CAC AGT CAG TGA 3’ for Slc1a2;

5’ TAG GAA CCT AAA TGG GAG GCT 3’ and 5’ CGC CAT TCC TGT GAC AAG AC 3’ for Slc1a3; 5’ ACT GCC TCT TTC TTC TTC CT 3’ and 5’ ATT TTC CCA AAC AAT GAC AC 3’ for Plp; 5’ AGA AAA CCG CAT CAC CAT T 3’ and 5’ CAT CTC CAC CGT CTT TAC CA 3’ for Gfap; 5’ GTG GAG AAC ACG GAT GAG AC 3’ and 5’ GAC ACA AGG TGG TTG AGG TC 3’ for Tubb3; 5’ AGC CTC AAG ATC ATC AGC AAT G 3’ and 5’ CAC GAT ACC AAA GTT GTC ATG GA 3’ for Gapdh. All the set of primers were designed with the help of Primer 3 program (Rozen and Skaletsky, 2000) according to the known sequences reported in GenBank.

Quantitative PCR was performed in a 36 well Rotor Gene 3000 (Corbett Research, Rotor-Gene™ 3000, version 5.0.60, Mortlake, Australia). For all the probands each cycle consisted of a denaturation step at 95°C for 15 s, followed by separate annealing (30 s) and extension (15 s) steps at a temperature characteristic for each proband. Fluorescence was monitored at the end of each extension step. A no-template, no-reverse transcriptase control was included in each experiment. At the end of the amplification cycles a melting curve analysis was added. The analysis of the data was made according to the Relative Standard Curve Method (Bustin, 2000). qRT-PCR data, expressed as the ratio between proband mRNA and Gapdh mRNA, are shown as the relative change with respect to the ratio obtained under control conditions (= 1).

2.7 Statistics

Both transport and qRT-PCR data were analyzed with a *t*-test for unpaired data.

2.8 Materials

Serum and culture medium were obtained from Celbio Euroclone (Milan, Italy). DL-*threo*-β-benzyloxyaspartate (DL-TBOA) was from Tocris Cookson (Bristol, UK). L-[2,3-^{3}H]Aspartic acid (39 Ci/mmol) was purchased from Amersham Pharmacia Biotech Italia, while D-[2,3-

^{3}H]aspartic acid (12 Ci/mmol) was obtained from Perkin Elmer (Monza, Italy). Sigma was the source of the other chemicals, including ATRA, whenever not stated otherwise.

Results

ATRA stimulates anionic amino acid transport in C6 cells

The results presented in Fig. 1, Panel A, demonstrate that the incubation of C6 cells at low serum in the presence of ATRA (10 μM) causes a marked increase in the transport of anionic amino acids. After 24h of incubation aspartate influx is already significantly higher in ATRA-treated than in cells incubated in the absence of the retinoid. ATRA-induced transport stimulation further increases from 24h to 48h of treatment and stabilizes thereafter. In contrast, during the same period, the transport activity of control cells decreases so that, after 4 days of treatment, aspartate transport is more than four-fold higher in ATRA-treated cultures than in control cells. A comparable effect of ATRA is also observed if C6 cells are maintained at 10% FBS (not shown). ATRA-dependent stimulation of aspartate transport is dose dependent (Fig. 1, Panel B), with a significant effect detectable at 100 nM. The dose response curve (not shown) yields an EC_{50} of 123 nM (R^2 = 0.992).

Under the conditions adopted, the incubation of C6 cultures in the presence of ATRA is associated with a modest, although significant, decrease of cell proliferation (Fig. 2, Panel A) and with marked changes in cell morphology (Panels B and C). In particular, flattened and spindle cells are common in control, untreated cultures (Panel B), while ATRA-treated cultures (Panel C) are mainly formed by cells characterized by two processes at the their extremities, a morphology previously named round bipolar (Ohira et al., 2006).

In both control and ATRA-treated cells, L-aspartate transport is accounted for by the additive activities of a single, saturable, high affinity mechanism and a non saturable, low affinity component formally undistinguishable from diffusion (Fig. 3, Panel A). The increase in aspartate transport is referable to a five-fold stimulation of the V_{max} of the saturable component (94 ± 30 vs. 519 ± 91 pmol/mg prot/min in control and ATRA-treated cells, respectively) while the K_m is not significantly different (11.4 ± 3.9 vs. 9.4 ± 2.2 μM). The

diffusion coefficient k_D is comparable in control and ATRA-treated cells (1.7 ± 0.12 vs. 1.5 ± 0.42 min^{-1}), thus excluding that the effect of the retinoid is to attribute to the stimulation of low affinity routes.

Figure 3, Panels B and C, reports the characterization of aspartate transport in control and ATRA-treated cells. For these experiments we have used the isomer D-aspartate, a specific substrate of excitatory amino acid transporters (EAAT) (Arriza et al., 1994). Under both conditions, the influx of D-aspartate is markedly lowered in the absence of sodium (more than 70% in control cells and more than 80% in ATRA-treated cells). The larger sodium dependence obtained in ATRA-treated cells is consistent with the larger portion of total aspartate influx referable to the high affinity, saturable route under the latter condition. The pre-incubation with PDBu, a treatment that triggers the PKC-dependent redistribution of EAAC1 to the plasma membrane (Fournier et al., 2004), causes an evident stimulation of aspartate transport in both control (Panel B) and ATRA-treated cells (Panel C). In both cell populations, aspartate transport was markedly inhibited by 2 mM L-aspartate, 2 mM D-aspartate, 1 mM DL-TBOA and L-cysteinsulphinate, indicating that it is due to a high affinity EAAT of the Slc1 family. Consistently, L-glutamate, but not D-glutamate, inhibits most of aspartate uptake in control and ATRA-treated cells, thus demonstrating that also upon ATRA-stimulation anionic amino acid transport exhibits the anomalous stereoselectivity that hallmarks EAAT (Gazzola et al., 1981). Under both conditions aspartate transport is not lowered by 25 μM dihydrokainate (DHK), a potent inhibitor of anionic amino acid transport through GLT-1 transporter. However, at 1 mM DHK partially inhibits aspartate transport in the two cell populations.

Changes in the expression of genes for EAAT transporters in ATRA-treated cells.

The predominant EAA transporter expressed by C6 cells is EAAC1, the product of Slc1a1 gene (Palos et al., 1996; Ma et al., 2006), although the expression of Slc1a2 and Slc1a3 is

also detectable at low levels in these cells (Imura et al., 1999). In order to assess if changes in the expression of a Slc1 gene are associated to ATRA-dependent stimulation of aspartate transport, we have performed a qRT-PCR analysis of the expression of Slc1a1-3 in C6 cells maintained for 4d in the absence or in the presence of 10 μM ATRA (Figure 4, Panel A). The results indicate that the relative expression of Slc1a1 mRNA, for EAAC1 transporter, markedly increases in the presence of ATRA. The increase in Slc1a1 expression is already significant after 24h of treatment, although it becomes more evident with treatment prolongation to reach a 4-fold stimulation after 4d of exposure to ATRA (Panel B). Slc1a2 mRNA, for GLT-1, is also significantly higher, although at a lesser degree, in ATRA-treated than in control cells, while, on the contrary, Slc1a3 mRNA, for GLAST transporter, is inhibited by ATRA treatment.

EAAC1 transporter expression is enhanced by ATRA

The results presented in Figure 5, Panel A, indicate that the abundance of EAAC1 protein is markedly enhanced by ATRA treatment, with a significant increase already detectable after 48h of treatment. Both the monomeric and the multimeric forms of EAAC1 are apparently increased in ATRA-treated cells. In contrast, cells incubated at low serum in the absence of the retinoid exhibit a progressive decrease of the carrier protein. As a result, after 4d of treatment EAAC1 abundance is 6-fold higher in ATRA-treated than in control cells. At this time, ATRA-treated cells have a much higher expression of EAAC1 than cells cultured under basal growth conditions (10% FBS, lane Ba). Neither GLAST nor GLT-1 transporters are detectable under the same conditions in which ATRA-induced EAAC1 expression is readily observed (Figure 5, Panel C).

The increase in EAAC1 expression in ATRA-treated cells is also evident in immunofluorescence (Fig. 6). Representative confocal sections of control and ATRA-treated cells, showing EAAC1 (Panels A and B), actin (Panels C and D), or the merged

signals (Panels E and F) demonstrate that high transporter expression occurs in most of the ATRA-treated cells (Panels B and F), whereas the level of expression in the control population (Panels A and E) is highly heterogeneous with some cells exhibiting a very low EAAC1 positivity. The quantitative analysis of the images indicates that the ratio of pixels positive for EAAC1 *vs.* those positive for actin increases from 0.40 to 1.53 in control and ATRA-treated cells, respectively. Moreover, under the same conditions, the mean intensity of EAAC1 signal rises from 55 to 82, while actin signal is comparable under the two conditions (52 *vs*. 53, in control and ATRA-treated cells, respectively). The highest EAAC1 signal is detectable in ATRA-treated cells with bipolar morphology. In both ATRA-treated and control cells, however, EAAC1 has a mainly intracellular localization.

ATRA treatment induces a marker of oligodendrocytic differentiation pathway.

It is known that ATRA treatment activates oligodendrocytic differentiation in C6 cells (Zhu et al., 1992; Lopez-Barahona et al., 1993), although this cell line is capable to express also astrocytic and neuronal markers under different experimental conditions (Segovia et al., 1994). To assess if EAAC1 induction is associated with the expression of specific differentiation markers under the experimental conditions adopted in this study, we have incubated C6 cells with 10 μM ATRA in the presence of 0.1% FBS and determined the relative expression of genes encoding markers of three differentiated phenotypes: proteolipid protein (Plp), a major myelin protein, for the oligodendrocytic pathway (Le Bras et al., 2005); glial fibrillary acidic protein (Gfap), for the astrocytic pathway (Kimelberg, 2004); and type III β-tubulin (Tubb3), for the neuronal pathway (Katsetos et al., 2003). The results, presented in Fig. 7, indicate that, after 4d of treatment, the expression of Plp is markedly enhanced in ATRA-treated cells, while Gfap and Tubb3 are lower than in cultures maintained in the absence of the retinoid. A significant PLP induction by ATRA is already detectable after 48h of treatment (results not shown).

Discussion

The repertoire of EAA transporters expressed in the central nervous system is exquisitely dependent upon the brain area, the cell type, the subcellular compartment considered, and the functional connections involved (Nieoullon et al., 2006; Beart and O'Shea, 2007). These considerations suggest that the expression of glutamate transporters is controlled by the same signals and mechanisms responsible for cell differentiation in CNS. However, little information is thus far available on this issue (Furuta et al., 1997a; Guillet et al., 2002; Schluter et al., 2002). Here we demonstrate that ATRA, an agent implied in the differentiation of central nervous system cells (Maden, 2007), has a clear cut stimulatory effect on the expression of the transporter EAAC1 transporter in C6 cells, a line derived from a rat glioma that contains neural stem cells (Kondo et al., 2004; Setoguchi et al., 2004; Zheng et al., 2007).EAAC1, encoded by Slc1a1, represents the predominant EAA transporter expressed by C6 cells (Palos et al., 1996; Ma et al., 2006), although the expression of Slc1a2 (for GLT-1 transporter) and Slc1a3 (for GLAST transporter) is also detectable at very low levels in these cells (Imura et al., 1999; Ma et al., 2006). Moreover, the expression of Slc1a2 by C6 cells is enhanced under hypertonic stress (Imura et al., 1999) and upon transfection with the proto-oncogene Wnt-1 (Palos et al., 1999), raising the alternative possibilities that the increase in aspartate transport associated with ATRA-induced differentiation is due to the overexpression of Slc1a1 or to the induction of a transporter normally present at low levels under control conditions, such as Slc1a2. The results presented in Fig. 4 indicate that the transport change is associated with increased levels of Slc1a1 mRNA but, under the same conditions, also the expression of Slc1a2 is increased, while Slc1a3 mRNA is lowered. However, at the protein level, only EAAC1, the product of Slc1a1, is markedly enhanced by ATRA, while no expression of GLT-1, the product of Slc1a2, is detected in both control and ATRA-treated cells. The absence of a detectable GLT-1 positivity may indicate that, although significantly stimulated by ATRA,

Slc1a2 expression is still too low to allow the production of significant levels of the carrier. Therefore, the ATRA-dependent increase in EAA transport should be referred to the overexpression of Slc1a1/EAAC1.

Consistently, the characterization of anionic amino acid transport (see Fig. 3B and C) indicates that the operative features of aspartate transport are the same in ATRA-treated and –untreated cells. In particular: (1) the transport process is mostly sodium-dependent, tolerates both L- and D-aspartate but interacts only weakly with the D isomer of glutamate and is suppressed by high affinity EAAT inhibitors such as DL-TBOA and L-cysteinsulphinate. (2) dihydrokainate does not inhibit aspartate uptake at 25 μM, a concentration corresponding to its K_i for GLT-1 (Arriza et al., 1994; Dowd et al., 1996), but lowers transport when used at 1 mM, a concentration comparable to its K_i for EAAC1 in C6 cells (Dowd et al., 1996); (3) phorbol esters cause transport stimulation, as expected for an EAAC1-dependent but not for a GLT-1-dependent process (Kalandadze et al., 2002; Namura et al., 2002; Gonzalez et al., 2005); . These features demonstrate that ATRA effects on aspartate transport are due to the stimulation of a typical EAA transporter endowed with the operational characteristics of EAAC1.

Among the high affinity transporters for glutamate expressed in the central nervous system, EAAC1 has several peculiar developmental characteristics. In particular, the transporter is characterized by an early expression during brain development (Bar-Peled et al., 1997; Furuta et al., 1997b; Guillet et al., 2002), when it is detectable before GLAST and GLT-1. A comparable expression profile has been recently demonstrated during the maturation of rat primary cortical cultures *in vitro* (Guillet et al., 2002). Moreover, EAAC1 is expressed in the radial glia during the development of CNS (Furuta et al., 2005), suggesting that the transporter is involved in the developmental effects of anionic amino acids on neurons (Nieoullon et al., 2006; Beart and O'Shea, 2007). Conversely, the transporter is down regulated during the astroglial differentiation of glial precursors

(Maragakis et al., 2004), an effect that may be connected to the slight inhibition of EAAC1 expression and activity observed in the present report during the prolonged low-serum culturing of C6 cells. However, in C6 cells ATRA does not simply prevent the decrease in EAAC1 expression and activity but, rather, causes a net increase of transporter abundance and transport activity, even compared to cells maintained at high serum (see Fig. 5). Moreover, ATRA effect should not be referred simply to the inhibition of cell proliferation, since (1) under the conditions adopted the antiproliferative effect of ATRA is modest (see Fig. 1, Panel A) and (2) ATRA-dependent induction of aspartate transport is observed also in high-serum medium (results not shown). More likely, the induction of EAAC1 is a part of the differentiation pathway triggered by ATRA treatment in C6 cells. Under different conditions and in different cell models, ATRA promotes the expression of neuronal, astrocytic and oligodendrocytic markers (Rutka et al., 1988; Sanchez-Ramos et al., 2001; Zhang et al., 2001; Jang et al., 2004) A preliminary analysis of three differentiation markers, all inducible by ATRA (Jang et al., 2004), indicates that, under the conditions adopted, ATRA treatment represses the expression of Gfap, an astrocytic marker, as well as of the neuronal marker Tubb3, while it greatly enhances the abundance of Plp mRNA, a marker of oligodendrocytic lineage. This result is consistent with data previously obtained in ATRA-treated C6 cells (Zhu et al., 1992; Lopez-Barahona et al., 1993) and indicates that the induction of EAAC1 may be associated with this differentiation pathway.

Oligodendrocytes are known to express EAAC1 *in vivo* (Conti et al., 1998), although they are also endowed with GLT-1 and GLAST “glial-type” transporters (Pitt et al., 2003; van Landeghem et al., 2007). In particular, results obtained by Matute’s group indicate that the expression of EAAC1 may be a characteristic of immature oligodendrocytes (Domercq et al., 1999), while mature cells would express GLT-1 and GLAST (Domercq et al., 1999; Vallejo-Illarramendi et al., 2006). Thus, ATRA-dependent EAAC1 induction may represent

a feature referable to the differentiation of an oligodendrocytic precursor. Interestingly, EAAC1 is induced in both neurons and oligodendrocytic precursors stressed by experimental ischemia (Gottlieb et al., 2000) while it is known that oligodendrocytes are sensitive to excitotoxicity (Yoshioka et al., 1996; Pitt et al., 2003) that, in particular, has been specifically described in oligodendrocytic precursors (Johnston, 2005). These data may point to a role of EAAC1 in the response of immature oligodendrocytes to excitotoxic conditions. In this context, it is interesting to note that ATRA-treated C6 cells exhibit a significantly higher resistance to excitotoxicity when pre-treated with sublethal doses of NMDA (Singh and Kaur, 2006). Although EAAC1 expression and activity were not investigated in that contribution, it is tempting to attribute, at least in part, this behaviour to the increased expression of the transporter.

EAAC1 activity and expression is highly regulated by intracellular signalling pathways involving protein kinase Cα (Gonzalez et al., 2003) and ε (Franchi-Gazzola et al., 1996), as well as phosphatidylinositol-3-kinase (Krizman-Genda et al., 2005). Most of these mechanisms act at post-translational level, changing the membrane trafficking of the transporter, while regulatory mechanisms acting at the level of Slc1a1 gene expression have been much less investigated thus far. In particular, while the stimulation of EAAC1/EAAT3 activity by ATRA has been described in a non nervous model (Butchbach et al., 2003), the stimulation could not be referred to changes in transporter expression. Thus, the results presented here represent the first demonstration of ATRA-dependent EAAC1 induction at both mRNA and protein level, although the identification of the precise site and mechanism of action of the retinoid will require additional experimental work.

Recently, transfection of C6 cells with the transcription factor RFX1 has been shown to induce EAAC1 expression (Ma et al., 2006). Although RFX1 is epigenetically silenced in several glioma cell lines (Ohashi et al., 2004), it is noteworthy that, in a non neural cell model, the expression of RFX1 protein is increased by ATRA treatment (Zajac-Kaye et al.,

2000). The possibility that RFX1 is involved in the ATRA-dependent induction of EAAC1 deserves, therefore, further investigation.

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References

Arriza JL, Fairman WA, Wadiche JI, Murdoch GH, Kavanaugh MP, Amara SG (1994) Functional comparisons of three glutamate transporter subtypes cloned from human motor cortex. J Neurosci 14:5559-5569.

Bar-Peled O, Ben-Hur H, Biegon A, Groner Y, Dewhurst S, Furuta A, Rothstein JD (1997) Distribution of glutamate transporter subtypes during human brain development. J Neurochem 69:2571-2580.

Beart PM, O'Shea RD (2007) Transporters for L-glutamate: an update on their molecular pharmacology and pathological involvement. Br J Pharmacol 150:5-17.

Benda P, Lightbody J, Sato G, Levine L, Sweet W (1968) Differentiated rat glial cell strain in tissue culture. Science 161:370-371.

Bustin SA (2000) Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J Mol Endocrinol 25:169-193.

Butchbach ME, Guo H, Lin CL (2003) Methyl-beta-cyclodextrin but not retinoic acid reduces EAAT3-mediated glutamate uptake and increases GTRAP3-18 expression. J Neurochem 84:891-894.

Conti F, DeBiasi S, Minelli A, Rothstein JD, Melone M (1998) EAAC1, a high-affinity glutamate tranporter, is localized to astrocytes and gabaergic neurons besides pyramidal cells in the rat cerebral cortex. Cereb Cortex 8:108-116.

de Toledo FG, Beers KW, Dousa TP (1997) Pleiotropic upregulation of Na(+)-dependent cotransporters by retinoic acid in opossum kidney cells. Am J Physiol 273:F438-444.

Dinsmore J, Ratliff J, Deacon T, Pakzaban P, Jacoby D, Galpern W, Isacson O (1996) Embryonic stem cells differentiated in vitro as a novel source of cells for transplantation. Cell Transplant 5:131-143.

Domercq M, Sanchez-Gomez MV, Areso P, Matute C (1999) Expression of glutamate transporters in rat optic nerve oligodendrocytes. Eur J Neurosci 11:2226-2236.

Dowd LA, Coyle AJ, Rothstein JD, Pritchett DB, Robinson MB (1996) Comparison of Na+-dependent glutamate transport activity in synaptosomes, C6 glioma, and Xenopus oocytes expressing excitatory amino acid carrier 1 (EAAC1). Mol Pharmacol 49:465-473.

Fischer I, Nolan CE, Shea TB (1987) Effects of retinoic acid on expression of the transformed phenotype in C6 glioma cells. Life Sci 41:463-470.

Fournier KM, Gonzalez MI, Robinson MB (2004) Rapid trafficking of the neuronal glutamate transporter, EAAC1: evidence for distinct trafficking pathways differentially regulated by protein kinase C and platelet-derived growth factor. J Biol Chem 279:34505-34513.

Franchi-Gazzola R, Visigalli R, Bussolati O, Gazzola GC (1996) Involvement of protein kinase Cepsilon in the stimulation of anionic amino acid transport in cultured human fibroblasts. J Biol Chem 271:26124-26130.

Furuta A, Martin LJ, Lin CL, Dykes-Hoberg M, Rothstein JD (1997a) Cellular and synaptic localization of the neuronal glutamate transporters excitatory amino acid transporter 3 and 4. Neuroscience 81:1031-1042.

Furuta A, Rothstein JD, Martin LJ (1997b) Glutamate transporter protein subtypes are expressed differentially during rat CNS development. J Neurosci 17:8363-8375.

Furuta A, Takashima S, Yokoo H, Rothstein JD, Wada K, Iwaki T (2005) Expression of glutamate transporter subtypes during normal human corticogenesis and type II lissencephaly. Brain Res Dev Brain Res 155:155-164.

Gazzola GC, Dall'Asta V, Bussolati O, Makowske M, Christensen HN (1981) A stereoselective anomaly in dicarboxylic amino acid transport. J Biol Chem 256:6054-6059.

Gonzalez MI, Bannerman PG, Robinson MB (2003) Phorbol myristate acetate-dependent interaction of protein kinase Calpha and the neuronal glutamate transporter EAAC1. J Neurosci 23:5589-5593.

Gonzalez MI, Susarla BT, Robinson MB (2005) Evidence that protein kinase Calpha interacts with and regulates the glial glutamate transporter GLT-1. J Neurochem 94:1180-1188.

Gottlieb M, Domercq M, Matute C (2000) Altered expression of the glutamate transporter EAAC1 in neurons and immature oligodendrocytes after transient forebrain ischemia. J Cereb Blood Flow Metab 20:678-687.

Gravel M, Gao E, Hervouet-Zeiber C, Parsons V, Braun PE (2000) Transcriptional regulation of 2',3'-cyclic nucleotide 3'-phosphodiesterase gene expression by cyclic AMP in C6 cells. J Neurochem 75:1940-1950.

Guillet B, Lortet S, Masmejean F, Samuel D, Nieoullon A, Pisano P (2002) Developmental expression and activity of high affinity glutamate transporters in rat cortical primary cultures. Neurochem Int 40:661-671.

Imura T, Shimohama S, Kageyama T, Kimura J (1999) Selective induction of glial glutamate transporter GLT-1 by hypertonic stress in C6 glioma cells. Biochem Biophys Res Commun 265:240-245.

Jang YK, Park JJ, Lee MC, Yoon BH, Yang YS, Yang SE, Kim SU (2004) Retinoic acid-mediated induction of neurons and glial cells from human umbilical cord-derived hematopoietic stem cells. J Neurosci Res 75:573-584.

Johnston MV (2005) Excitotoxicity in perinatal brain injury. Brain Pathol 15:234-240.

Kalandadze A, Wu Y, Robinson MB (2002) Protein kinase C activation decreases cell surface expression of the GLT-1 subtype of glutamate transporter. Requirement of a carboxyl-terminal domain and partial dependence on serine 486. J Biol Chem 277:45741-45750.

Kanai Y, Hediger MA (2003) The glutamate and neutral amino acid transporter family: physiological and pharmacological implications. Eur J Pharmacol 479:237-247.

Katsetos CD, Legido A, Perentes E, Mork SJ (2003) Class III beta-tubulin isotype: a key cytoskeletal protein at the crossroads of developmental neurobiology and tumor neuropathology. J Child Neurol 18:851-866; discussion 867.

Kimelberg HK (2004) The problem of astrocyte identity. Neurochem Int 45:191-202.

Kondo T, Setoguchi T, Taga T (2004) Persistence of a small subpopulation of cancer stem-like cells in the C6 glioma cell line. Proc Natl Acad Sci U S A 101:781-786.

Krizman-Genda E, Gonzalez MI, Zelenaia O, Robinson MB (2005) Evidence that Akt mediates platelet-derived growth factor-dependent increases in activity and surface expression of the neuronal glutamate transporter, EAAC1. Neuropharmacology 49:872-882.

Le Bras B, Chatzopoulou E, Heydon K, Martinez S, Ikenaka K, Prestoz L, Spassky N, Zalc B, Thomas JL (2005) Oligodendrocyte development in the embryonic brain: the contribution of the plp lineage. Int J Dev Biol 49:209-220.

Lind CR, Gray CW, Pearson AG, Cameron RE, O'Carroll SJ, Narayan PJ, Lim J, Dragunow M (2006) The mitogen-activated/extracellular signal-regulated kinase kinase 1/2 inhibitor U0126 induces glial fibrillary acidic protein expression and reduces the proliferation and migration of C6 glioma cells. Neuroscience 141:1925-1933.

Lopez-Barahona M, Minano M, Mira E, Iglesias T, Stunnenberg HG, Rodriguez-Pena A, Bernal J, Munoz A (1993) Retinoic acid posttranscriptionally up-regulates proteolipid protein gene expression in C6 glioma cells. J Biol Chem 268:25617-25623.

Ma K, Zheng S, Zuo Z (2006) The transcription factor regulatory factor X1 increases the expression of neuronal glutamate transporter type 3. J Biol Chem 281:21250-21255.

Maden M (2007) Retinoic acid in the development, regeneration and maintenance of the nervous system. Nat Rev Neurosci 8:755-765.

Maragakis NJ, Dietrich J, Wong V, Xue H, Mayer-Proschel M, Rao MS, Rothstein JD (2004) Glutamate transporter expression and function in human glial progenitors. Glia 45:133-143.

McMorris FA (1983) Two independent mechanisms of induction of 2':3'-cyclic-nucleotide 3'-phosphohydrolase in glioma cells by cyclic AMP and high cell density. Biochim Biophys Acta 762:560-568.

Namura S, Maeno H, Takami S, Jiang XF, Kamichi S, Wada K, Nagata I (2002) Inhibition of glial glutamate transporter GLT-1 augments brain edema after transient focal cerebral ischemia in mice. Neurosci Lett 324:117-120.

Nieoullon A, Canolle B, Masmejean F, Guillet B, Pisano P, Lortet S (2006) The neuronal excitatory amino acid transporter EAAC1/EAAT3: does it represent a major actor at the brain excitatory synapse? J Neurochem 98:1007-1018.

Ohashi Y, Ueda M, Kawase T, Kawakami Y, Toda M (2004) Identification of an epigenetically silenced gene, RFX1, in human glioma cells using restriction landmark genomic scanning. Oncogene 23:7772-7779.

Ohira K, Homma KJ, Hirai H, Nakamura S, Hayashi M (2006) TrkB-T1 regulates the RhoA signaling and actin cytoskeleton in glioma cells. Biochem Biophys Res Commun 342:867-874.

Palos TP, Ramachandran B, Boado R, Howard BD (1996) Rat C6 and human astrocytic tumor cells express a neuronal type of glutamate transporter. Brain Res Mol Brain Res 37:297-303.

Palos TP, Zheng S, Howard BD (1999) Wnt signaling induces GLT-1 expression in rat C6 glioma cells. J Neurochem 73:1012-1023.

Parker KK, Norenberg MD, Vernadakis A (1980) "Transdifferentiation" of C6 glial cells in culture. Science 208:179-181.

Pflug BR, Colangelo AM, Tornatore C, Mocchetti I (2001) TrkA induces differentiation but not apoptosis in C6-2B glioma cells. J Neurosci Res 64:636-645.

Pitt D, Nagelmeier IE, Wilson HC, Raine CS (2003) Glutamate uptake by oligodendrocytes: Implications for excitotoxicity in multiple sclerosis. Neurology 61:1113-1120.

Rozen S, Skaletsky H (2000) Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol 132:365-386.

Rutka JT, De Armond SJ, Giblin J, McCulloch JR, Wilson CB, Rosenblum ML (1988) Effect of retinoids on the proliferation, morphology and expression of glial fibrillary acidic protein of an anaplastic astrocytoma cell line. Int J Cancer 42:419-427.

Sanchez-Ramos JR, Song S, Kamath SG, Zigova T, Willing A, Cardozo-Pelaez F, Stedeford T, Chopp M, Sanberg PR (2001) Expression of neural markers in human umbilical cord blood. Exp Neurol 171:109-115.

Schluter K, Figiel M, Rozyczka J, Engele J (2002) CNS region-specific regulation of glial glutamate transporter expression. Eur J Neurosci 16:836-842.

Segovia J, Lawless GM, Tillakaratne NJ, Brenner M, Tobin AJ (1994) Cyclic AMP decreases the expression of a neuronal marker (GAD67) and increases the expression of an astroglial marker (GFAP) in C6 cells. J Neurochem 63:1218-1225.

Setoguchi T, Taga T, Kondo T (2004) Cancer stem cells persist in many cancer cell lines. Cell Cycle 3:414-415.

Singh J, Kaur G (2006) HSP70 induction and oxidative stress protection mediated by a subtoxic dose of NMDA in the retinoic acid-differentiated C6 glioma cell line. Brain Res Bull 69:37-47.

Takanaga H, Yoshitake T, Hara S, Yamasaki C, Kunimoto M (2004) cAMP-induced astrocytic differentiation of C6 glioma cells is mediated by autocrine interleukin-6. J Biol Chem 279:15441-15447.

Vallejo-Illarramendi A, Domercq M, Perez-Cerda F, Ravid R, Matute C (2006) Increased expression and function of glutamate transporters in multiple sclerosis. Neurobiol Dis 21:154-164.

van Landeghem FK, Weiss T, von Deimling A (2007) Expression of PACAP and glutamate transporter proteins in satellite oligodendrocytes of the human CNS. Regul Pept.

Vanhoutte N, de Hemptinne I, Vermeiren C, Maloteaux JM, Hermans E (2004) In vitro differentiated neural stem cells express functional glial glutamate transporters. Neurosci Lett 370:230-235.

Yoshioka A, Bacskai B, Pleasure D (1996) Pathophysiology of oligodendroglial excitotoxicity. J Neurosci Res 46:427-437.

Zajac-Kaye M, Ben-Baruch N, Kastanos E, Kaye FJ, Allegra C (2000) Induction of Myc-intron-binding polypeptides MIBP1 and RFX1 during retinoic acid-mediated differentiation of haemopoietic cells. Biochem J 345 Pt 3:535-541.

Zhang WL, Tsuneishi S, Nakamura H (2001) Induction of heat shock proteins and its effects on glial differentiation in rat C6 glioblastoma cells. Kobe J Med Sci 47:77-95.

Zheng X, Shen G, Yang X, Liu W (2007) Most C6 cells are cancer stem cells: evidence from clonal and population analyses. Cancer Res 67:3691-3697.

Zhu W, Kanoh M, Ye P, Laszkiewicz I, Royland JE, Wiggins RC, Konat G (1992) Retinoic acid-regulated expression of proteolipid protein and myelin-associated glycoprotein genes in C6 glioma cells. J Neurosci Res 31:745-750.

Legends to figures

Fig. 1. ATRA increases aspartate transport in C6 cells. Panel A. After seeding, C6 cells were incubated for the indicated times with fresh medium supplemented with 0.1% FBS in the absence (■) or in the presence of 10 μM ATRA (▲). Aspartate transport was measured (see Experimental Procedures) at the indicated times. Panel B. Before transport assay, cells were incubated for 3 days in the presence of the indicated concentrations of ATRA. Data represent the portion of aspartate transport due to the activity of high-affinity transporters, calculated subtracting the uptake measured in the presence of 2 mM L-Asp from the total uptake. For both panels, data are means of five independent determinations with SD shown when greater than the point size. ** $p < 0.01$ and *** $p < 0.001$, vs. control, untreated cells.

Fig. 2. Effects of ATRA on cell proliferation and morphology. Panel A. Cultures of C6 glioma cells were seeded at a density of $10 \cdot 10^3$ cells/cm^2 in complete growth medium. After 12h, medium was replaced with fresh medium supplemented with 0.1% FBS in the absence (■) or in the presence of 10 μM ATRA (▲). Viability was assessed at the indicated times with resazurin assay (see Experimental Procedures). Data are means of five independent determinations with SD shown when greater than the point size. ** $p < 0.01$ and *** $p < 0.001$, vs. control, untreated cells. Panels B and C. Microphotographs of C6 cultures incubated for 4d at 0.1% FBS in the absence (Panel B) or in the presence of 10 μM ATRA (Panel C). Bar = 50 μm.

Fig. 3. Characterization of ATRA-stimulated aspartate transport. C6 cells were incubated for 4d in the absence or in the presence of 10 μM ATRA, as indicated. Panel A. The influx

of L-aspartate concentrations ranging from 1 to 256 μM was determined as described in Experimental Procedures. Solid lines represent best fits of data to Eqn. 1 (see Experimental Procedures, R^2 0.982 and 0.916 for control and ATRA-treated cells, respectively). The dotted and the dashed line represent the saturable component of aspartate influx for control and ATRA-treated cells, respectively. Panels B and C. The influx of 1 μM D-aspartate (1 μM, 2 μCi/ml) was determined in cells incubated for 4d in culture medium supplemented with either 0.1% FBS (Panel B) or 0.1 FBS + 10 μM ATRA (Panel C). Transport assay was performed under the following conditions, as indicated: replacement of EBSS with sodium-free EBSS during the assay; pre-incubation with 150 nM PDBu for 15 min; presence of 2 mM L-aspartate, 2 mM D-aspartate, 1 mM DL-TBOA, 2 mM L-glutamate, 2 mM D-glutamate, 2 mM L-cysteinsulphinate (L-CSS), 25 μM DHK or 1 mM DHK during the assay period. Values of transport under control conditions were 15 ± 3.2 and 68 ± 9.1 pmol/mg prot/min for ATRA-untreated and ATRA-treated cells, respectively.

For the three panels, points are means of 5 independent determinations with SD shown when greater of the size of the point. For Panels B and C, *$p < 0.05$, **$p < 0.01$, ***p < 0.001 vs. control (transport determined in unsupplemented Earle's Balanced Salt Solution).

Fig. 4. Induction of Slc1a1 mRNA by ATRA. Panel A. C6 cells were incubated for 4d in the absence or in the presence of 10 μM ATRA, as indicated. After RNA extraction and reverse transcription, cDNA was employed as template for qPCR with Slc1a1, Slc1a2, Slc1a3, or Gapdh primers (see Experimental Procedures). The intensities of the Slc1 products were normalized to that of Gapdh product amplified from the same cDNA sample. The mean Ct values under control conditions (FBS 0.1%) were: 12.1 for Gapdh, 20.1 for Slc1a1, 23.8 for Slc1a2 and 29.3 for Slc1a3. Panel B. Cells were incubated for the

indicated times in the absence or in the presence of 10 μM ATRA. Slc1a1 mRNA was processed and quantified as described in Panel A. For the two panels, data are means ± SD of two separate experiments, each performed in duplicate. *$p < 0.05$, **$p < 0.01$, ATRA-treated vs. control, untreated cells.

Fig. 5. EAAC1 protein is overexpressed in ATRA-treated C6 cells. Panel A. Time-course of EAAC1 expression in ATRA-treated C6 cells. EAAC1 and GAPDH expression were analyzed in lysates of C6 cells incubated for the indicated times in growth medium supplemented with FBS 0.1% in the absence or in the presence of 10 μM ATRA. Lane Ba (Basal) contains the lysate of C6 cells obtained before the substitution of high-serum with low-serum medium. Panel B reports the results of the densitometric analysis of the blot shown in Panel B. Panel C. Western Blot of GLAST (left), GLT1 (middle) and EAAC1 (right) in C6 cells. Lanes 1, 4, 7, rat brain extract ; lanes 2, 5, 8, lysates of C6 cells incubated for 4d at 0.1% FBS in the absence of ATRA; lanes 3, 6, 9, lysates of C6 cells incubated for 4d at 0.1% FBS in the presence of 10 μM ATRA. The experiments were repeated twice with comparable results.

Fig. 6. Immunocytochemical analysis of EAAC1 expression in C6 cells. Immunocytochemistry was performed, as described under Experimental Procedures, on C6 cells seeded on coverslips and incubated for 4d in the absence (Panels A, C, E) or in the presence of 10 μM ATRA (Panels B, D, F). Panels A and B, EAAC1 expression (red). Panels C and D, Actin (green). Panels E and F show the merge images. Arrows, bipolar cells. A single confocal section of a representative field is shown. Bar = 20 μm. Palettes are shown in Panel A.

Fig. 7. Expression of differentiation markers in ATRA-treated C6 cells. C6 cells were incubated for 4d at 0.1% FBS in the absence or in the presence of 10 μM ATRA, as indicated. After RNA extraction and reverse transcription, cDNA was used as a template for qPCR with Plp, Gfap, Tubb3 or Gapdh primers (Experimental Procedures. The intensity of proband product was normalized to that of Gapdh product amplified from the same cDNA sample. Data are means ± SD of two separate experiments, each performed in duplicate. *$p < 0.05$, **$p < 0.01$, ATRA-treated vs. control, untreated cells.

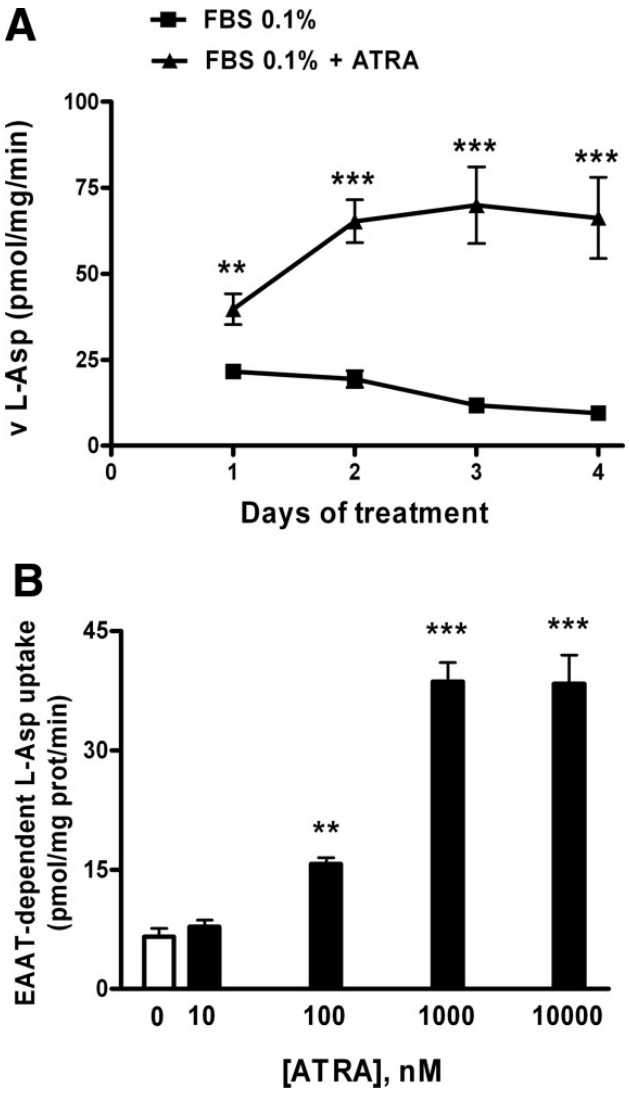
A
FBS 0.1%
FBS 0.1% + ATRA
v L-Asp (pmol/mg/min)
100
75
50
25
0
0
1
2
3
4
Days of treatment
**
***
***
***
B
EAAT-dependent L-Asp uptake (pmol/mg prot/min)
45
30
15
0
0
10
100
1000
10000
[ATRA], nM
**
***
***

Figure 1

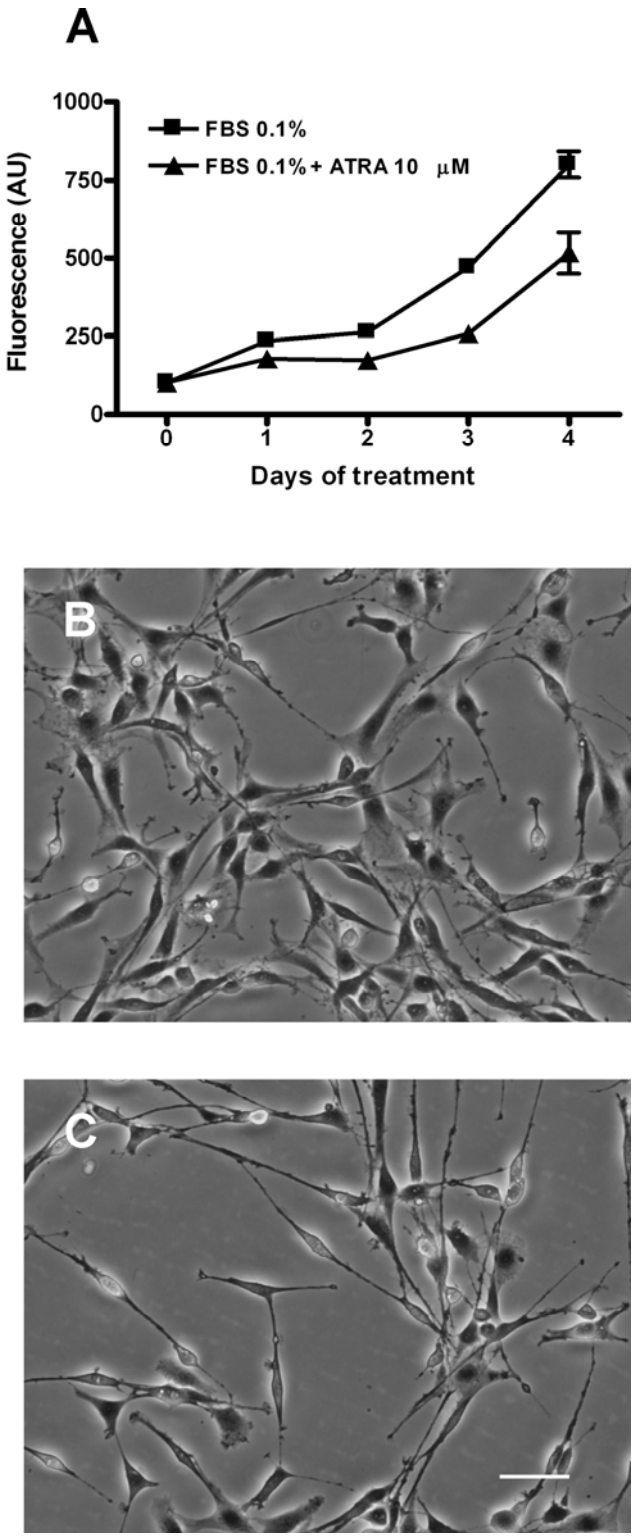
A
1000
750
500
250
0
Fluorescence (AU)
FBS 0.1%
FBS 0.1% + ATRA 10 μM
0
1
2
3
4
Days of treatment
B
C

Figure 2

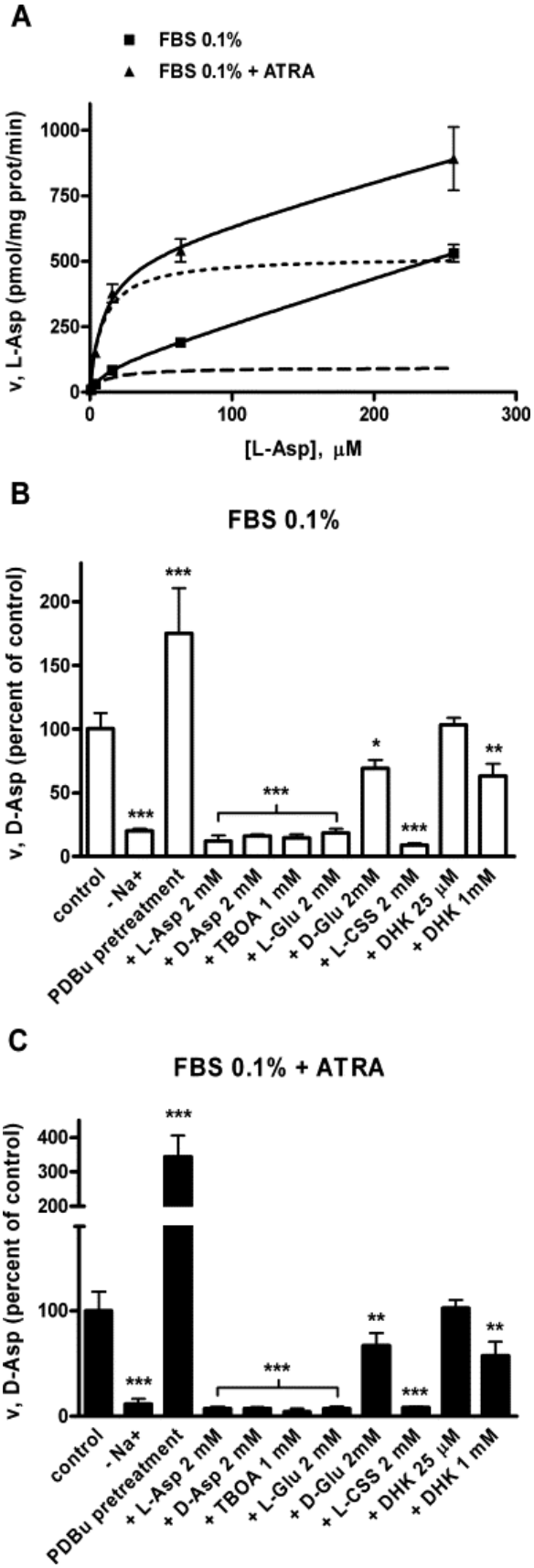
A
FBS 0.1%
FBS 0.1% + ATRA
v, L-Asp (pmol/mg prot/min)
1000
750
500
250
0
0
100
200
300
[L-Asp], µM
B
FBS 0.1%
v, D-Asp (percent of control)
200
150
100
50
0
***
***
***
*
***
**
control
- Na+
PDBu pretreatment
+ L-Asp 2 mM
+ D-Asp 2 mM
+ TBOA 1 mM
+ L-Glu 2 mM
+ D-Glu 2mM
+ L-CSS 2 mM
+ DHK 25 µM
+ DHK 1mM
C
FBS 0.1% + ATRA
v, D-Asp (percent of control)
400
300
200
100
0
***
***
***
**
***
**
control
- Na+
PDBu pretreatment
+ L-Asp 2 mM
+ D-Asp 2 mM
+ TBOA 1 mM
+ L-Glu 2 mM
+ D-Glu 2mM
+ L-CSS 2 mM
+ DHK 25 µM
+ DHK 1 mM

Figure 3

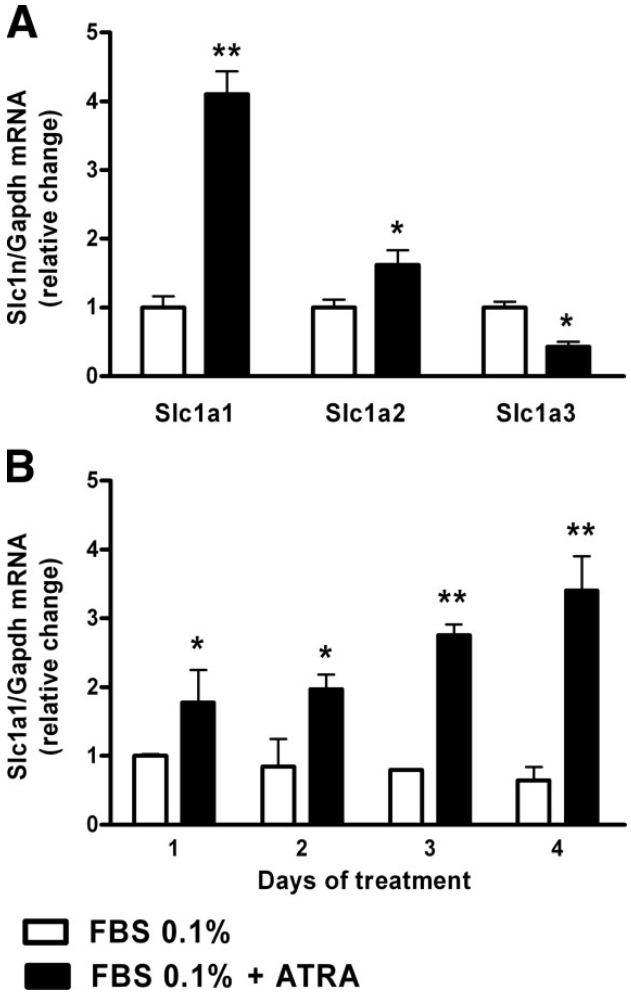
A
Slc1n/Gapdh mRNA
(relative change)
5
4
3
2
1
0
**
*
*
Slc1a1
Slc1a2
Slc1a3
B
Slc1a1/Gapdh mRNA
(relative change)
5
4
3
2
1
0
*
*
**
**
1
2
3
4
Days of treatment
FBS 0.1%
FBS 0.1% + ATRA

Figure 4

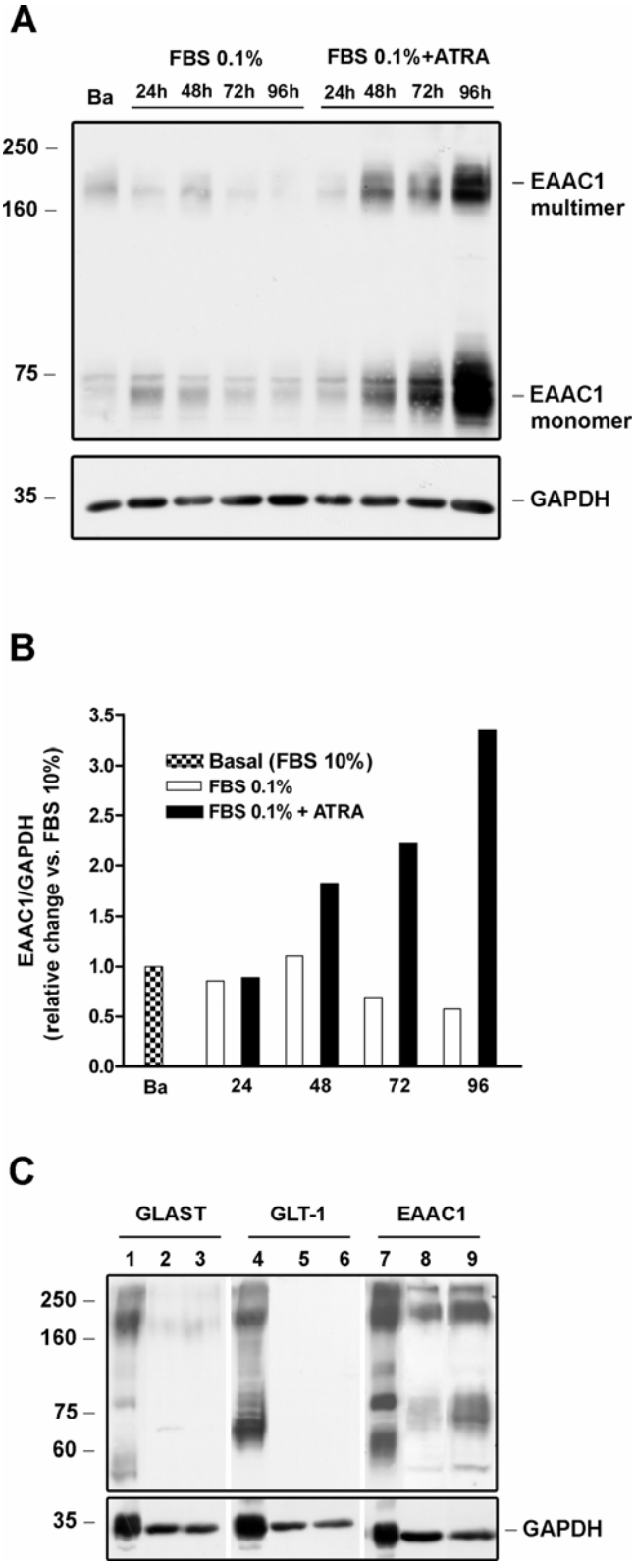
A
FBS 0.1%
FBS 0.1%+ATRA
Ba
24h 48h 72h 96h
24h 48h 72h 96h
250
160
75
35
EAAC1 multimer
EAAC1 monomer
GAPDH
B
EAAC1/GAPDH (relative change vs. FBS 10%)
Basal (FBS 10%)
FBS 0.1%
FBS 0.1% + ATRA
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Ba
24
48
72
96
C
GLAST
GLT-1
EAAC1
1 2 3
4 5 6
7 8 9
250
160
75
60
35
GAPDH

Figure 5

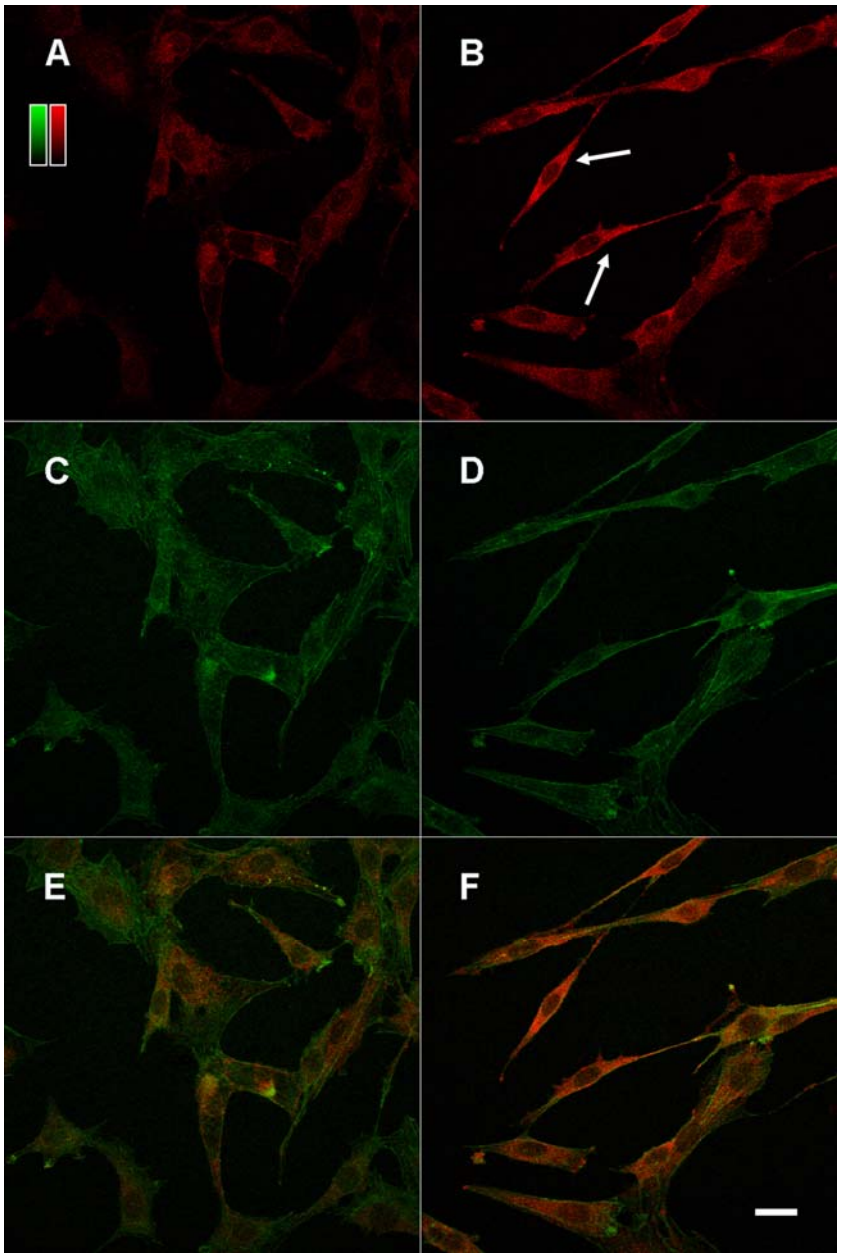

Figure 6

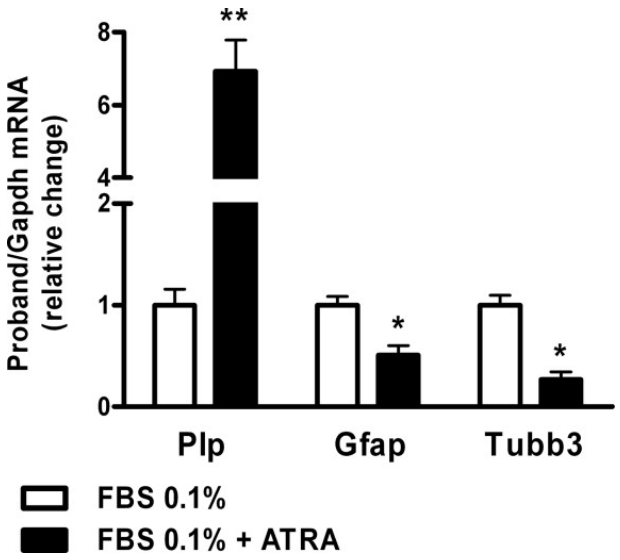
Proband/Gapdh mRNA
(relative change)
8
6
4
2
1
0
**
*
*
Plp
Gfap
Tubb3
FBS 0.1%
FBS 0.1% + ATRA

Figure 7

It is known that all-*trans* retinoic acid (ATRA) plays a pivotal role in the development and regeneration of the CNS (for a recent review see (Maden, 2007). In particular, ATRA can induce differentiation of $CD133^+$ hematopoietic stem cells toward neuron, astrocyte and oligodendrocyte lineage. (Dinsmore et al., 1996; Sanchez-Ramos et al., 2001; Jang et al., 2004). Moreover, several studies have been carried out to address the hypothesis regarding the regulation of glutamate transporters by ATRA. For example, ATRA seems to augment both the EAAT2/GLT-1 and EAAT1/GLAST expression in neuronal stem cells.

In ATRA-treated cells we observed for the first time a huge increase in glutamate transport activity due by an increase of the Slc1a1 gene expression as well as of the EAAC1/EAAT3 protein expression (Bianchi et al., 2008). Although this report cannot be taken as an example of a pharmacological tool for the promotion of Slc1a1 gene expression, it may represent a starting point in this direction.

Moreover, since Slc1a1 induction is associated with the overexpression of a marker of oligodendrocyte lineage, EAAC1 may play a role in some step of that differentiation pathway. This hypothesis has been preliminarily investigated in ex vivo models (see Conclusions).

A recent paper indicates that the Slc1a1 gene expression is enhanced in C6 cells transfected with human RFX-1b gene sequences (Ma et al., 2006). Consistently, the knockdown of RFX-1 in rat cortical neurons in culture decreased the EAAC1 expression suggesting that RFX-1 may be a putative transcription factor for Slc1a1 also *in vivo*, raising the possibility that ATRA-induced changes in the Slc1a1 are mediated by RFX1. However, ATRA may influence gene expression through many different pathways. To investigate these possibilities we have undertaken an experimental work to define the molecular mechanisms underlying ATRA effect (Bianchi et al., submitted, 2009b).

The ATRA-dependent overexpression of the glutamate transporter EAAC1 requires RARβ induction

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Running title: Mechanism of retinoic effect on glutamate transport

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ABSTRACT

Several mechanisms modulate the activity of EAAC1, the counterpart of the human EAAT3 carrier, through changes in transporter trafficking and interaction with regulatory proteins. Much less is known on the regulation of Slc1a1, the gene that encodes for the transporter. We have recently found that all-*trans* retinoic acid (ATRA) markedly induces Slc1a1 expression and EAAC1 activity in C6 rat glioma cells (Bianchi M.G. et al, Neuroscience 151 (2008) 1042-1052). We report here that ATRA effects on EAAC1 activity were inhibited by the specific RAR inhibitor LE540 and mimicked by AM80, a RAR agonist, but not by the RXR agonist HX631. In addition, Slc1a1 mRNA induction was suppressed by cycloheximide, suggesting that a protein intermediate is needed for ATRA effect. The half-life of Slc1a1-mRNA was comparable to that of Gapdh in control cells and markedly shorter in ATRA-treated cells, suggesting that the retinoid did not increase the messenger half-life. ATRA treatment induced the expression of Rarb mRNA several hours before the induction of Slc1a1 while the mRNA for RFX1, a transcription factor recently involved in Slc1a1 transcription, was unchanged. Moreover, silencing of Rarb, while suppressed ATRA-induced mRNA of the receptor, markedly inhibited also the induction of the transporter. We conclude that in C6 glioma cells the induction of Slc1a1 by ATRA requires the previous synthesis of RARβ, suggesting that the transporter gene is a target of this receptor.

Key words: EAAT3; C6 Glioma; Retinoids; RXR; Slc1a1; Transport.

ABBREVIATIONS

The abbreviations used are: ATRA, all-*trans*-retinoic acid; DMEM, Dulbecco's modified Eagle Medium; EAAC1, Excitatory Amino Acid Carrier 1; EAAT, Excitatory Amino Acid Transporter; EBSS, Earle's Balanced Salt Solution; FBS, Fetal Bovine Serum.

The glutamate transporter EAAT3 and its murine counterpart EAAC1, although present in several cell types, are mainly expressed in SNC neurons [1]. As other members of the EAAT family, EAAC1, which is encoded by the Slc1a1 gene, has been involved in the regulation of the extracellular glutamate concentration and in synaptic plasticity [2]. Alterations in the activity and/or expression of the transporter have been implied in several neurological and psychiatric conditions [3]. For these reasons, the regulation of EAAC1/EAAT3 activity has been investigated in a number of studies (see [3] for a recent review). Most of these involve rapid mechanisms that modify transporter trafficking between the plasma membrane and intracellular compartments, where most of the carrier proteins are located [4].

Much less is known about EAAC1/EAAT3 regulation at the gene level. Ma et al [5] have shown that the transfection of rat glioma C6 cells with the human transcription factor RFX1 induces EAAC1 expression. More recently, we have found that in the same cell model the chronic exposure to all-trans retinoic acid (ATRA) causes a marked induction of Slc1a1, the gene that encodes for EAAC1, causing the increased abundance of carrier proteins and the proportional stimulation of transport activity [6]. Under the same conditions, Slc1a1 induction is associated with the increased expression of Plp, a marker of the oligodendrocytic differentiation pathway and a well known ATRA target [7]. The induction of Plp mRNA by ATRA in C6 cells is a slow phenomenon, requires a protein intermediate and has been attributed to an increase in the half-life of the messenger [8].

A variety of genes are direct or indirect targets of retinoids and their expression is modulated by ATRA at both transcriptional and post-transcriptional level. In particular, the transcription of direct ATRA targets is promoted by a complex formed by ATRA, an heterodimer of RAR-RXR receptors and several other co-stimulator molecules. Indirect regulation of gene expression by ATRA comprises various mechanisms such as (a) the induction of an intermediary (usually a

transcription factor) that is the actual responsible for the effect, (b) the modulation of mRNA stability, and (c) the interaction with nuclear receptors other than the classical RAR-RXR dimers [9]. Moreover, retinoids can affect transcription through non genomic effects, such as modulations of cAMP or MAPK cascade [10].

The essential step required for the direct transcriptional effects of ATRA is represented by the interaction of the retinoid with the heterodimer formed by a member of RAR receptor family (RARα, β, γ, see [11] for review) and a RXR receptor (RXRα, β, γ, [12]). Upon the interaction with the ligand, the heterodimer exchanges co-repressor partners with co-activator partners and the transcription of the target gene can start. While RARα is expressed in most tissues[12], the expression of RARβ and RARγ is tissue specific and highly regulated [13]. Interestingly, two of the RARβ isoforms, RARβ2 and 4, are transcribed from a promoter that contains a DR5 sequence and, hence, are inducible by retinoids [14, 15]. Consistently, ATRA treatment markedly affects the expression of RARβ receptors in many cell types. In particular, in C6 cells ATRA stimulates the expression of one of the “heavy”, 55 kDa isoform of RARβ, while RARα is not affected by the retinoid [16].

In this report we have studied the mechanisms underlying the effect of the retinoid on EAAC1, obtaining evidences that involve the synthesis and the activation of a RARβ receptor as one of the steps of the regulatory process.

METHODS AND MATERIALS

Cells.

The rat central nervous system-derived C6 glioma cells were obtained from American Type Culture Collection (Rockville, MD). Cells were grown at 37°C and 5% CO2 in Dulbecco's modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 4 mM glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin. Cells were used for less than 10 passages from thawing with no apparent change in morphology or sensitivity to treatments.

For ATRA treatment, unless otherwise specified, culture medium was substituted with fresh medium supplemented with 0.1% FBS in the absence or in the presence of ATRA 12h after the passage. ATRA, obtained from Sigma, was used at the concentrations detailed for each experiment starting from a 10 mM stock solution in DMSO.

Transport activity.

The initial influx of D-[^{3}H]aspartate, an high-affinity substrate of excitatory amino acid (EAA) transporters (Kanai and Hediger, 2003), was measured in 96-well multidish plates (Falcon, Becton, Dickinson Biosciences, Franklin Lakes, NJ, USA), where C6 cells had been seeded at a density of $10 \cdot 10^3$ cells/well, or in Falcon 24-well multidish plates, where cells had been seeded at a density of $50 \cdot 10^3$ cells/well. For the experiments, cells were washed twice in a modified EBSS (Earle's Balanced Salt Solution, containing (in mM) 117 NaCl, 5 KCl, 1.8 $CaCl_2$, 1 NaH_2PO_4, 0.8 $MgSO_4$, 5.5 glucose, buffered with 20 mM HEPES-NaOH at pH 7.4) and incubated in the same saline solution supplemented with D-[^{3}H]aspartate (1 µM, 2µCi/ml) for 1 min.

At the end of the assay, multiwell dishes were washed twice with ice-cold urea (300 mM) and cell monolayers were extracted with absolute ethanol. The extracts were added to scintillation fluid and counted with a Wallac Trilux^2 liquid scintillation spectrometer (Perkin-Elmer, Boston, MA, USA). Cell proteins were determined directly in the well with a modified Lowry procedure and measured with a Wallac Victor^2 Multilabel Counter (Perkin-Elmer). Amino acid influx is expressed as pmoles·mg of protein^{-1}·min^{-1}.

RT-qPolymerase Chain Reaction

Total RNA was isolated with RNeasy Mini Kit® (Qiagen S.p.a., Milan, Italy). After reverse transcription, 25 ng of cDNA from each sample was amplified in a total volume of 25 µl with 2X SYBRGreen qPCR SuperMix-ROX (Rovalab, Taltow, Germany), along with the following forward and reverse primers (5 pmol each) for the genes: 5' CGA CTT GCC GTA CCT GGA CT 3' and 5' GCC CAC GGG ACT CAA CAC GA 3' for Slc1a1; 5' ATA CCC CAG AGC AAG ACA CC 3' and 5' AGC AGA TGG CAC TGA GAA GA 3' for Rarb; 5' AAC CAG AGA GCC GAT TTT AGA G 3' and 5' AAC TGT TGC TAC CCA CCC TAC T 3' for Rfx1; 5' AGC CTC AAG ATC ATC AGC AAT G 3' and 5' CAC GAT ACC AAA GTT GTC ATGGA 3' for Gapdh. All the set of primers were designed with the help of Primer 3 program (Rozen and Skaletsky, 2000) according to the known or predicted (Rfx1) sequences reported in GenBank. Quantitative PCR was performed in a 36 well Rotor Gene 3000 (Corbett Research, Rotor-Gene™ 3000, version 5.0.60, Mortlake, Australia). For all the probands each cycle consisted of a denaturation step at 95°C for 30 s, followed by separate annealing (30 s) and extension (30 s) steps at a temperature characteristic for each proband. Fluorescence was monitored at the end of each extension step. A no template, no-reverse transcriptase control was included in each experiment. At the end of the amplification cycles a melting curve analysis was

added. The analysis of the data was made according to the Relative Standard Curve Method (Bustin, 2000). qRT-PCR data, expressed as the ratio between proband mRNA and Gapdh mRNA,

Western analysis

C6 cells, grown to subconfluence on 10-cm tissue culture plates, were rinsed twice in PBS and lysed in 300 µl of Sample Buffer 1x (31.25 mM Tris-HCl pH 6.8, 3% SDS, 10% glycerol, 100 mM DTT, 0.02% bromophenol blu). After solubilization, cell lysates were collected in eppendorf tubes and passed 10 times into 25G and then 27 G needles. The quantification of proteins was performed with a modified Lowry assay (Ref.) and aliquots of 60 µg were loaded on a 10% gel for SDS-PAGE. After the electrophoresis, proteins were transferred to nitrocellulose membranes (Schleicher & Schuell Bioscience, Dassel, Germany). Non-specific binding sites were blocked with an incubation in PBS-Tween 0.1% containing 5% of non fat dried milk (Amersham Pharmacia Biotech Italia, Milan, Italy) for 2h at room temperature. The blots were then exposed overnight at 4°C to anti-RARβ1-2 (1:250) or anti RARα polyclonal antisera (1:200), obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), diluted in blocking solution. After washing, the blots were exposed for 1h to horseradish peroxidase-conjugated anti-rabbit IgG (ExactaCruz, Santa Cruz Biotechnology) diluted 1:10000 in blocking solution. For the standardization of the total cell lysate and the intracellular fraction, stripped membranes were exposed to monoclonal antiserum against GAPDH (Chemicon International Inc., Temecula, CA, USA, 1:1000). The membranes were washed and the immunoreactivity visualized with enhanced chemiluminescence (Millipore, Billerica, MA).

Silencing experiments

Silencing of Rarb mRNA was carried out in C6 cells using the N-TER^{TM} Nanoparticle siRNA Transfection System from Sigma-Aldrich. The same manufacturer provided also the oligonucleotides against Rrab1-2 (oligos #3834795 and #3834796 (siRNA1); oligos #3834797 and #3834798 (siRNA2); oligos #3834799 and #3834800 (siRNA3)) and scrambled siRNAs (oligos #3834801 and #383802). The preparation of oligonucleotide/peptide mixtures was performed according to manufacturer's instructions.

The protocols used for Rarb silencing are detailed in the legends of the single experiments (Fig. 6). According to the first protocol, once the mixtures were ready, culture medium was replaced with serum-free medium containing anti-Rarb siRNA/peptide or scrambled siRNA/peptide complexes at a doubled concentration and incubation prolonged for further 4h. At this time, an equal volume of medium supplemented with 20% FBS was added to each culture so as to reach the desired final concentration of the siRNA, specified in the single experiments, in medium supplemented with 10% FBS for the following 20h. After this period, cells did not show evident signs of toxicity. After silencing, medium was renewed again with a fresh, siRNA-free medium, supplemented with 0.1% FBS, in the absence or in the presence of ATRA 1 μM. mRNA was extracted after 16h.

Statistical analysis.

Statistical analysis of transport data was performed with an one way ANOVA with the Bonferroni post hoc test. qRT-PCR data were analyzed with a *t*-test for unpaired data (Fig. 4) or with an one way ANOVA with the Bonferroni test for selected pairs of conditions (Figs. 2 and 7). In all cases p values > 0.05 were considered not significant.

Materials.

FBS and culture medium were purchased from Celbio, (Pero, MI, Italy). D-[2,3-3H]aspartic acid (12 Ci/mmol) was obtained from Perkin Elmer (Monza, Italy). Sigma was the source of the other chemicals, including ATRA, whenever not stated otherwise.

RESULTS AND DISCUSSION

EAAC1 induction is inhibited by RAR but not by RXR inhibitors and is mimicked by RAR agonists

To investigate the mechanism underlying Slc1a1 induction in ATRA-treated cells we examined the effects of agonists or antagonists of RAR and RXR receptors on the ATRA-dependent stimulation of EAAC1 transport activity. The results of this analysis, recounted in Figure 1, indicate that the specific Retinoic Acid Receptor (RAR) agonist Am80 mimicked the maximal ATRA effect, while the RXR agonist HX630 was ineffective (Panel A). However, cells treated simultaneously with Am80 and HX630 exhibited a transport activity for aspartate significantly higher than cells treated with 1 μM ATRA. Panels B and C report the results obtained with antagonists. These experiments, in which ATRA was used at low, sub-maximal concentrations, indicated that the specific RAR antagonist LE540 significantly inhibited the effect of 50 nM ATRA and completely suppressed transport stimulation at 20 nM ATRA (Panel B). On the contrary, ATRA effect was not inhibited by 1 μM HX 531, a RXR antagonist that also inhibits RARs at high concentrations, but was significantly lowered when the inhibitor was used at 2.5 μM (Panel C). In summary, these results indicate that the ATRA-dependent stimulation of EAAC1 transport activity requires the interaction of the retinoid with a RAR receptor.

Slc1a1 induction requires protein synthesis and does not involve changes in messenger half-life

ATRA-dependent stimulation of EAAC1 activity is associated with the increase in the level of Slc1a1 mRNA [6]. To investigate the mechanism underlying this effect we have performed ATRA treatment in the presence of the protein synthesis inhibitor cycloheximide and

compared the behaviour of the transporter messenger with that of other two ATRA target mRNAs, Plp, which encodes for the proteolipid protein, and Rarb, encoding for the RARβ receptor (Figure 2). The results obtained indicate that the inhibitor completely suppressed the increase in Slc1a1 levels caused by ATRA, demonstrating that ATRA-dependent stimulation of Slc1a1 expression requires the new synthesis of a protein. The requirement for active protein synthesis is shared by other, but not by all, ATRA target genes. Indeed, the mRNA for RARβ was still significantly increased by ATRA even in the presence of cycloheximide while ATRA-dependent induction of Plp mRNA was not detectable under the same conditions (see also [8]). Interestingly, both Rarb and Plp mRNAs were significantly induced by cycloheximide even in the absence of ATRA.

ATRA-dependent increase of Plp mRNA is particularly interesting since it is an example of ATRA effect at post-transcriptional level, being mediated through changes in the half-life of the messenger [8]. The possibility that the same mechanism operates in ATRA-dependent stimulation of Slc1a1 expression has been investigated blocking transcription in control cells or in cells treated with ATRA for 24h. The results (Figure 3) indicate that in ATRA-treated cells the ratio of Slc1a1 and Gapdh mRNAs was not statistically different in control and ATRA-treated cells. The behaviour of Rarb mRNA is comparable, while, as expected [8], the ratio of Plp and Gapdh mRNAs increased progressively indicating that the half-life of Plp messenger is longer in the presence of ATRA than in its absence.

Slc1a1 induction is forerun by RARβ but not by RFX induction

The transfection of C6 cells with the human transcription factor RFX1 has been recently reported to activate Slc1a1 expression [5]. Interestingly, RFX1 protein is induced by ATRA in human myeloid models [17]. Thus, although no direct evidence is available about ATRA sensitivity of Rfx1 gene, the possibility exists that the protein intermediate required for Slc1a1 induction is RFX1. In order to verify this hypothesis, we have performed a short-term time course of Slc1a1 and Rfx1 mRNAs during ATRA treatment (Figure 4). The results indicate that, while a significant Slc1a1 induction is detected at 16h of treatment as well as at longer times (Panel A), Rfx1 is not consistently induced during ATRA treatment (Panel B). Rather, a moderate decrease in the expression levels of the transcription factor is detected both in the absence and in the presence of the retinoid. Consistently, no increase in RFX1 protein is detected during ATRA treatment up to 72h of treatment (data not shown).

As expected from literature [8], a rapid, ATRA-dependent induction of RARβ is instead detectable from the first time of treatment (4h) and still observed, although at lower levels, at the longest time examined (48h, Panel C). The fast induction of RARβ under our experimental conditions has been confirmed at protein level, through a Western Blot that, moreover, indicated that the induction of the receptor was an early change induced by ATRA. The primary antibody used in this experiment recognizes all the RARβ isoforms and, consistently, at least two bands are induced during the treatment while a third band, already present under control conditions, is greatly enhanced (Figure 5, Panel A). The densitometric analysis, shown in Panel B, indicates that the maximal expression of the receptor is detected at 4h to decrease at later times, although it is still clearly detectable at 24h. Under the same conditions no changes in RARα expression were detected either at mRNA or protein level (not shown).

Slc1a1 induction is inhibited by RARβ silencing

To evaluate the role of RARβ in ATRA effects on Slc1a1 we used a silencing approach aimed to interfere with Rarb induction by ATRA. We preliminarily tested three siRNAs (1, 2, 3) targeted to Rarb1-2 mRNAs at two different concentrations and found that scrambled siRNA did not affect ATRA-dependent Rarb-induction which, instead, was markedly affected by one of the three anti-Rarb siRNAs used (siRNA-2). Rarb silencing by siRNA-2 was dose dependent, with a 70% inhibition of ATRA-dependent induction observed with a concentration of 50 nM (Fig. 6). The inhibitions observed with the other two siRNAs were smaller and poorly dose-dependent. Attempts to use combinations of low concentrations of two siRNAs or prolongation of ATRA treatment over 24h yielded smaller inhibitions than those observed with siRNA-2 alone and a 24h-ATRA treatment (not shown).

We then tested the effects of siRNA-2 on Slc1a1 induction by ATRA. In these experiments we had to perform ATRA treatment for 24h so as to ensure a significant Slc1a1 induction. To maintain an effective silencing for this relatively long time, we decided to prolong the exposure to siRNA throughout ATRA treatment, raising the FBS concentration to10% and ATRA concentration to 10 μM (see the legend to Figure 7 for the experimental details). Under these conditions Rarb-silenced cells exhibited an effective inhibition of both Rarb and Slc1a1 ATRA-dependent induction (Figure 7A and 7B). In cells treated with scrambled siRNA ATRA increased Slc1a1 expression by 140% (Figure 7B), while the modest induction observed in Rarb-silenced cells (35%) was not significant.

CONCLUSIONS

ATRA is a major regulator of gene expression in eukaryotes and the expression of several hundreds of genes is affected by this retinoid. However, only a portion of these genes are directly activated by the ATRA according to the classical mechanism of binding and activation of a heterodimeric complex of RAR-RXR receptors. In many other cases, ATRA works indirectly through several mechanisms, the best known of which involve the synthesis of transcription factors that, conversely, target gene transcription or cause changes in messenger half-life. The results recounted in this contribution imply RARβ as the mediator of ATRA effects on EAAC1 transporter. This conclusion is based on several lines of evidence.

First, the pharmacological characterization of ATRA-dependent stimulation of performed strongly suggest that one or more receptors of the RAR family are involved since the RAR-specific agonist AM80 fully mimicked ATRA effect. Interestingly, under the same conditions, the synthetic rexinoid HX630 does not stimulates aspartate transport, but, in its presence, AM80 produces a greater influx stimulation than in its absence. This synergistic effect may be explained given that RXRs are unable to respond to rexinoids (RXR-selective agonists) in the absence of RAR ligand, since, under this condition, dissociation of corepressors from the heterodimer does not occur [18]. However, the rexinoid receptor is not transcriptionally silent since RAR activation produces larger biological effects in the presence of a RXR ligand than in its absence, as originally demonstrated by Lotan et al. for the inhibition of cancer cell proliferation [19] and, more recently, by Idres et al for the induction of CYP26A1 [20].

Second, RARb is induced before the stimulation of Slc1a1 expression, a finding consistent with its role of a transcription factor needed for the synthesis of the transporter. Indeed, cycloheximide suppresses Slc1a1 mRNA induction, indicating that a protein intermediate is needed for the effect, which may be either at transcriptional or at post-transcriptional level.

However, ATRA treatment does not change the half life of Slc1a1 compared with that of the housekeeping, ATRA-independent Gapdh gene. On the contrary, under the same conditions, the post-transcriptional effect of ATRA on messenger half-life, described in C6 cells for several ATRA target genes, such as Plp [8], β1-adrenergic receptor [21] and connexin 43 [22], is readily observed here as an increase in Plp mRNA half-life under the same conditions. It is, therefore, likely that the protein intermediate required for Slc1a1 induction is a factor acting at the transcriptional level. A possible candidate is RFX1, a factor found to stimulate Slc1a1 expression when transfected in C6 cells [5]. However, Rfx1 is not stimulated during ATRA treatment at both mRNA and protein level thus rendering unlikely its involvement in ATRA-dependent Slc1a1 regulation. On the contrary, Rarβ is a typical, direct ATRA target gene [9] and, consistently with data in literature [8], RARβ induction is readily detected at early times of ATRA-treatment of C6 cells at mRNA level. Moreover, also RARβ proteins are greatly induced at early treatment times so that C6 cells are endowed with an increased abundance of these receptors when Slc1a1 expression is stimulated.

Third, and more importantly, Rarb silencing markedly inhibits the induction of the transporter while the induction of another ATRA target gene, Plp, is less affected. The silencing approach adopted here is quite effective, producing a marked fall in Rarb mRNA levels both in ATRA-treated and in control cells. In particular, Rarb induction is suppressed by almost 70%. The inhibitory effect on Slc1a1 is comparable, indicating a strong relationship between RARβ expression and Slc1a1 induction. The specificity of the inhibition is demonstrated by the smaller effect of the same protocol on the induction of Plp (not shown).

In conclusion, this report points to Slc1a1 as a RARβ-dependent ATRA target gene. Further experiments are needed to identify which RARβ isoform is involved in the stimulation of

EAAC1 expression as well as which are the ATRA response elements present in the Slc1a1 gene.

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REFERENCES

[1] Y. Kanai, M.A. Hediger, The glutamate and neutral amino acid transporter family: physiological and pharmacological implications, Eur J Pharmacol 479 (2003) 237-247.

[2] A.V. Tzingounis, J.I. Wadiche, Glutamate transporters: confining runaway excitation by shaping synaptic transmission, Nat Rev Neurosci 8 (2007) 935-947.

[3] P.M. Beart, R.D. O'Shea, Transporters for L-glutamate: an update on their molecular pharmacology and pathological involvement, Br J Pharmacol 150 (2007) 5-17.

[4] W. Yang, M.S. Kilberg, Biosynthesis, intracellular targeting, and degradation of the EAAC1 glutamate/aspartate transporter in C6 glioma cells, J Biol Chem 277 (2002) 38350-38357.

[5] K. Ma, S. Zheng, Z. Zuo, The transcription factor regulatory factor X1 increases the expression of neuronal glutamate transporter type 3, J Biol Chem 281 (2006) 21250-21255.

[6] M.G. Bianchi, G.C. Gazzola, L. Tognazzi, O. Bussolati, C6 glioma cells differentiated by retinoic acid overexpress the glutamate transporter excitatory amino acid carrier 1 (EAAC1), Neuroscience 151 (2008) 1042-1052.

[7] W. Zhu, M. Kanoh, P. Ye, I. Laszkiewicz, J.E. Royland, R.C. Wiggins, G. Konat, Retinoic acid-regulated expression of proteolipid protein and myelin-associated glycoprotein genes in C6 glioma cells, J Neurosci Res 31 (1992) 745-750.

[8] M. Lopez-Barahona, M. Minano, E. Mira, T. Iglesias, H.G. Stunnenberg, A. Rodriguez-Pena, J. Bernal, A. Munoz, Retinoic acid posttranscriptionally up-regulates proteolipid protein gene expression in C6 glioma cells, J Biol Chem 268 (1993) 25617-25623.

[9] J.E. Balmer, R. Blomhoff, Gene expression regulation by retinoic acid, J Lipid Res 43 (2002) 1773-1808.

[10] P. Lefebvre, P.J. Martin, S. Flajollet, S. Dedieu, X. Billaut, B. Lefebvre, Transcriptional activities of retinoic acid receptors, Vitam Horm 70 (2005) 199-264.

[11] P. Germain, P. Chambon, G. Eichele, R.M. Evans, M.A. Lazar, M. Leid, A.R. De Lera, R. Lotan, D.J. Mangelsdorf, H. Gronemeyer, International Union of Pharmacology. LX. Retinoic acid receptors, Pharmacol Rev 58 (2006) 712-725.

[12] P. Germain, P. Chambon, G. Eichele, R.M. Evans, M.A. Lazar, M. Leid, A.R. De Lera, R. Lotan, D.J. Mangelsdorf, H. Gronemeyer, International Union of Pharmacology. LXIII. Retinoid X receptors, Pharmacol Rev 58 (2006) 760-772.

[13] M. Mark, N.B. Ghyselinck, P. Chambon, Function of retinoid nuclear receptors: lessons from genetic and pharmacological dissections of the retinoic acid signaling pathway during mouse embryogenesis, Annu Rev Pharmacol Toxicol 46 (2006) 451-480.

[14] P. Dolle, E. Ruberte, P. Leroy, G. Morriss-Kay, P. Chambon, Retinoic acid receptors and cellular retinoid binding proteins. I. A systematic study of their differential pattern of transcription during mouse organogenesis, Development 110 (1990) 1133-1151.

[15] A. Zelent, C. Mendelsohn, P. Kastner, A. Krust, J.M. Garnier, F. Ruffenach, P. Leroy, P. Chambon, Differentially expressed isoforms of the mouse retinoic acid receptor beta generated by usage of two promoters and alternative splicing, EMBO J 10 (1991) 71-81.

[16] P. Reboul, P. George, P. Louisot, P. Broquet, Study of retinoic acid effect upon retinoic acid receptors beta (RAR-beta) in C6 cultured glioma cells, Biochem Mol Biol Int 36 (1995) 1097-1105.

[17] M. Zajac-Kaye, N. Ben-Baruch, E. Kastanos, F.J. Kaye, C. Allegra, Induction of Myc-intron-binding polypeptides MIBP1 and RFX1 during retinoic acid-mediated differentiation of haemopoietic cells, Biochem J 345 Pt 3 (2000) 535-541.

[18] S. Westin, R. Kurokawa, R.T. Nolte, G.B. Wisely, E.M. McInerney, D.W. Rose, M.V. Milburn, M.G. Rosenfeld, C.K. Glass, Interactions controlling the assembly of nuclear-receptor heterodimers and co-activators, Nature 395 (1998) 199-202.

[19] R. Lotan, M.I. Dawson, C.C. Zou, L. Jong, D. Lotan, C.P. Zou, Enhanced efficacy of combinations of retinoic acid- and retinoid X receptor-selective retinoids and alpha-interferon in inhibition of cervical carcinoma cell proliferation, Cancer Res 55 (1995) 232-236.

[20] N. Idres, J. Marill, G.G. Chabot, Regulation of CYP26A1 expression by selective RAR and RXR agonists in human NB4 promyelocytic leukemia cells, Biochem Pharmacol 69 (2005) 1595-1601.

[21] M. Lopez-Barahona, T. Iglesias, I. Garcia-Higuera, F. Mayor, Jr., A. Zaballos, J. Bernal, A. Munoz, Post-transcriptional induction of beta 1-adrenergic receptor by retinoic acid, but not triiodothyronine, in C6 glioma cells expressing thyroid hormone receptors, Eur J Endocrinol 135 (1996) 709-715.

[22] X. Zhang, Z. Ren, J. Zuo, C. Su, R. Wang, Y. Chang, F. Fang, The effect of all-trans retinoic acid on gap junctional intercellular communication and connexin 43 gene expression in glioma cells, Chin Med Sci J 17 (2002) 22-26.

LEGENDS TO FIGURES

Figure 1. Effect of RAR and RXR agonists and antagonists on ATRA-dependent increase of EAAC1 activity. C6 cells were seeded in 96-well (Panel A) or in 24-well multiwell dishes (Panels B and C). After 24h, cells were incubated in the absence (control, empty bars) or in the presence of the indicated agonists or antagonists (solid bars), as detailed in each panel. Transport of D-Asp was measured after 3 days. Bars are means ± SD of five (Panel A) or three (Panels B and C) independent determinations in representative experiments, repeated twice with comparable results. For all panels, ** $p < 0.01$, *** $p < 0.001$ vs. control cells; ns, not significant vs. control cells; for panel A, ### $p < 0.001$ vs. cells treated with ATRA and vs. cells treated with 1 μM Am580; for panel B, ### $p < 0.001$ vs. cells treated with ATRA and vs. cells treated with 50 nM ATRA, $$$ $p < 0.001$ vs. cells treated with 20 nM ATRA; for panel C, ### $p < 0.001$ vs. cells treated with 1 μM Hx531, § $p < 0.05$ vs. cells treated with 50 nM ATRA.

Figure 2. Dependence of Slc1a1 induction upon protein synthesis. C6 cells were incubated for 3 days in the absence or in the presence of 10 μM ATRA and of 5 μM cycloheximide. At the end of this period, the relative abundance of Slc1a1, Plp, and Rarb mRNA was determined with RT-PCR. Data are means ± SD of four determinations obtained in two different experiments. * $p < 0.05$, ** $p < 0.01$, $p < 0.001$ vs. respective control, ATRA-untreated cells; ns, not significant vs. respective, ATRA-untreated controls.

Figure 3. ATRA effects on the half life of Slc1a1, Rarb and Plp mRNAs. C6 cells were incubated for 24h in the absence or in the presence of 10 μM ATRA. After this period, 2.4 μM actinomycin D was added to the extracellular medium and incubation prolonged, with or without ATRA, for further 12h. At the indicated times, RNA was extracted and the expression of the indicated genes (Panel A, Slc1a1; Panel B, Rarb; Panel C, Plp) was determined through RT-PCR. Points are means of four determinations obtained in two distinct experiments. Lines are the best fit linear regressions. The slopes of the regression lines obtained in the absence and in the presence of ATRA were significantly different ($p < 0.01$) only for Plp (Panel C).

Figure 4. ATRA effects on gene expression in C6 cells. C6 cells were incubated for the indicated times in the absence or in the presence of 10 μM ATRA. At the indicated times, RNA was extracted and the expression of the indicated genes (Panel A, Slc1a1; Panel B, Rfx1; Panel C, Rarb) was determined through RT-PCR. Data are expressed relatively to Gapdh mRNA abundance and are means of four determinations obtained in two distinct experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control, ATRA-untreated cells extracted at the same experimental time.

Figure 5. Induction of RARβ by ATRA in C6 cells, C6 cells were incubated for the indicated times in the absence or in the presence of 1 μM ATRA. Cells were then lysed and the lysates examined for the expression of RARβ. GAPDH expression was used as a loading control. The lower part of the Figure reports the densitometric analysis of the results. The experiment was repeated twice with comparable results.

Figure 6. Effects of anti-Rarb-siRNA on the ATRA-induced expression of Rarb. The culture medium of C6 cells was replaced with medium containing 10% FBS and the anti-Rarb siRNA/peptide or scrambled siRNA/peptide complexes (as indicated, see Methods). After 24h, medium was renewed again with a fresh, siRNA-free medium, supplemented with 0.1% FBS, in the absence or in the presence of ATRA 1 μM. mRNA was extracted after 8h and the abundance of Rarb and Gapdh mRNAs was determined with RT-PCR. Data of Rarb mRNA abundance are expressed relatively to Gapdh mRNA and are means of two determinations with SD in a representative experiment. The experiment was repeated twice with comparable results.

Figure 7. Effect of RARβ silencing on the ATRA-induced expression of Slc1a1. The culture medium of C6 cells was replaced with serum free medium containing the anti-Rarb siRNA/peptide or scrambled siRNA, both at a concentration of 100 nM, and the incubation was prolonged for 4h. An equal volume of medium supplemented with 20% FBS was then added to each culture so as to lower the siRNA concentration at 50 nM and to raise the FBS concentration to 10%. After 20h, ATRA (10 μM) or vehicle were added to the incubation medium. mRNA was extracted after further 24h of incubation and the abundance of Rarb (Panel A) and Slc1a1 (Panel B) was determined with RT-PCR. Data represent means of three determinations in a representative experiment repeated twice with comparable results.

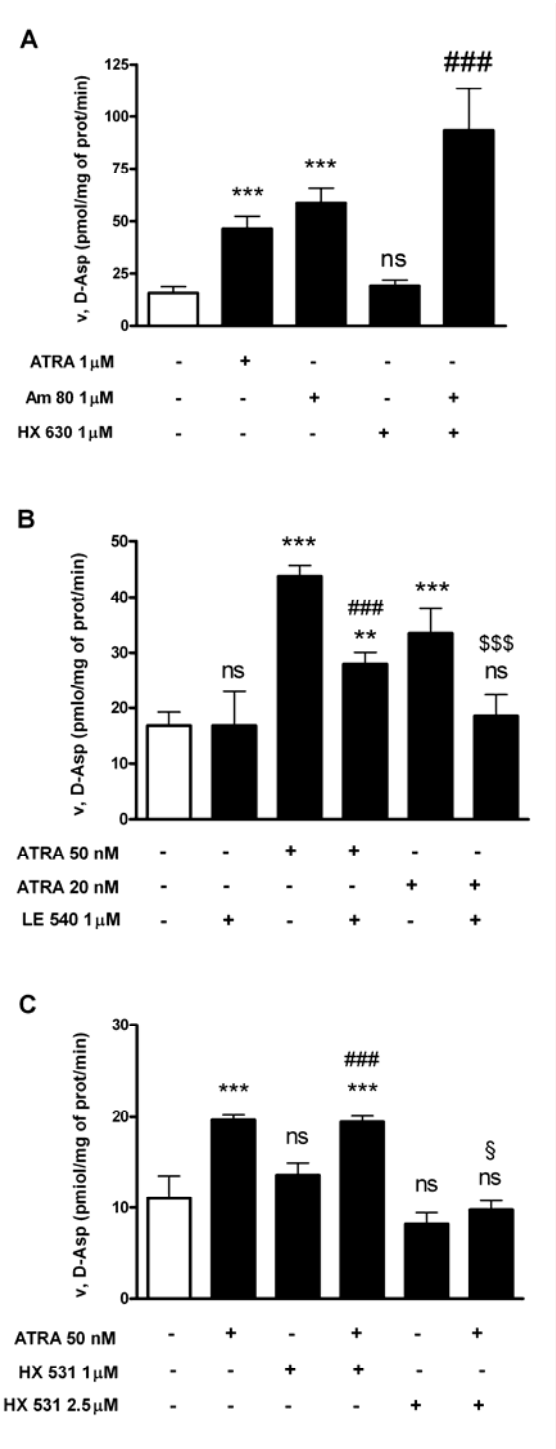
A
v, D-Asp (pmol/mg of prot/min)
125
100
75
50
25
0
***
***
ns
###
ATRA 1 μM - + - - -
Am 80 1 μM - - + - +
HX 630 1 μM - - - + +
B
v, D-Asp (pmlo/mg of prot/min)
50
40
30
20
10
0
ns
***
###
**
***
$$$
ns
ATRA 50 nM - - + + - -
ATRA 20 nM - - - - + +
LE 540 1 μM - + - + - +
C
v, D-Asp (pmiol/mg of prot/min)
30
20
10
0
***
ns
###
***
ns
§
ns
ATRA 50 nM - + - + - +
HX 531 1 μM - - + + - -
HX 531 2.5 μM - - - - + +

Figure 1

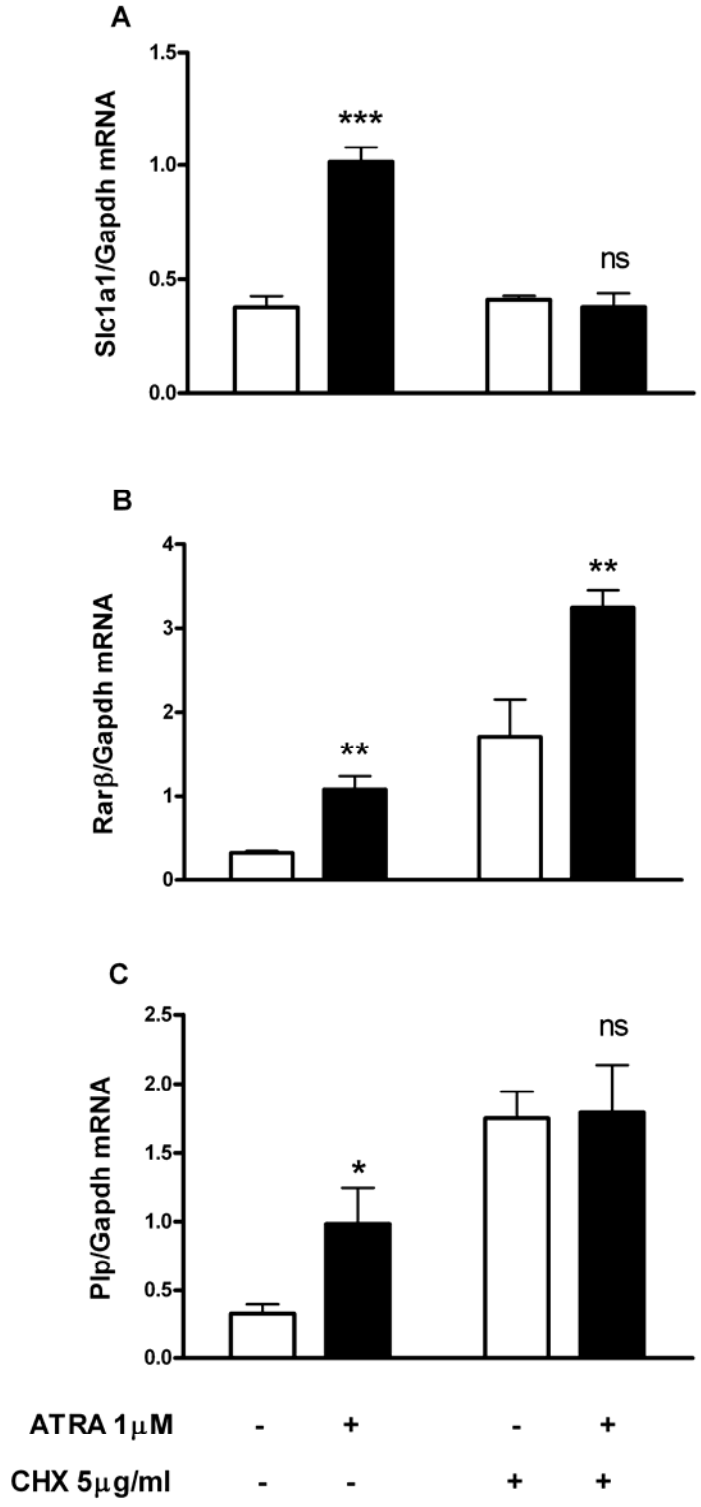
A
Slc1a1/Gapdh mRNA
1.5
1.0
0.5
0.0
***
ns
B
Rarβ/Gapdh mRNA
4
3
2
1
0
**
**
C
Plp/Gapdh mRNA
2.5
2.0
1.5
1.0
0.5
0.0
*
ns
ATRA 1μM - + - +
CHX 5μg/ml - - + +

Figure 2

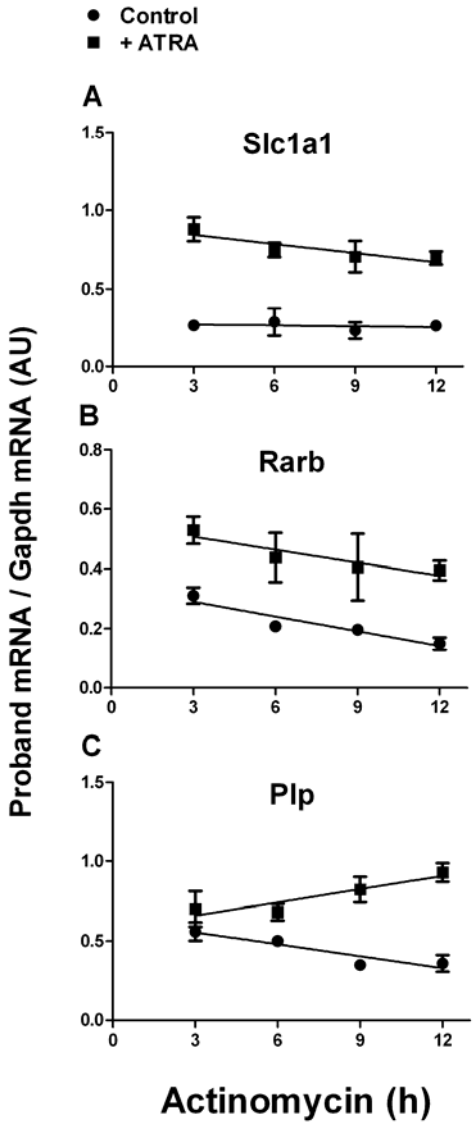
Control
+ ATRA
A
Slc1a1
B
Rarb
C
Plp
Proband mRNA / Gapdh mRNA (AU)
Actinomycin (h)

Figure 3

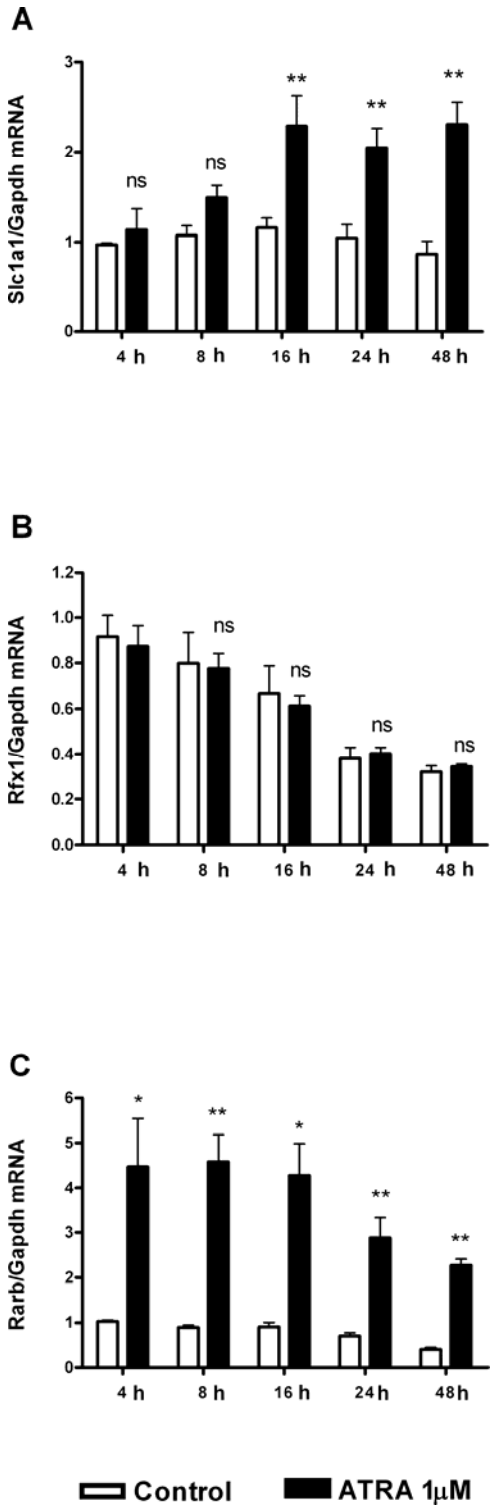
A
Slc1a1/Gapdh mRNA
3
2
1
0
ns
ns
**
**
**
4 h
8 h
16 h
24 h
48 h
B
Rfx1/Gapdh mRNA
1.2
1.0
0.8
0.6
0.4
0.2
0.0
ns
ns
ns
ns
4 h
8 h
16 h
24 h
48 h
C
Rarb/Gapdh mRNA
6
5
4
3
2
1
0
*
**
*
**
**
4 h
8 h
16 h
24h
48h
Control
ATRA 1μM

Figure 4

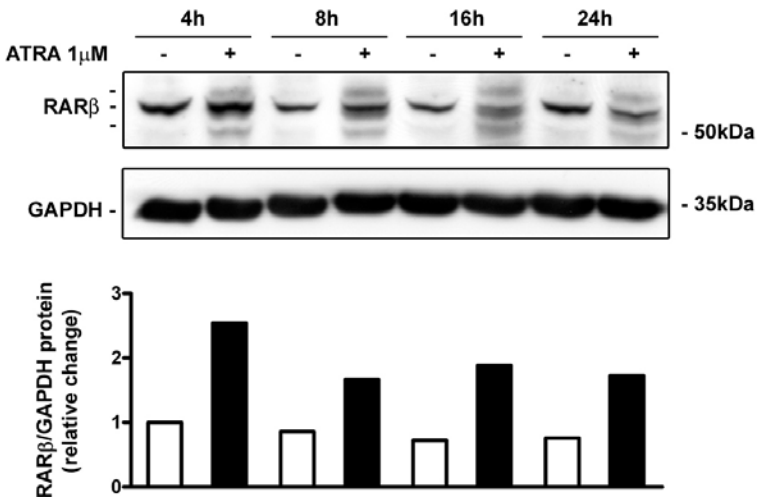
4h
8h
16h
24h
ATRA 1μM
- + - + - + - +
RARβ
- 50kDa
GAPDH -
- 35kDa
RARβ/GAPDH protein (relative change)
3
2
1
0

Figure 5

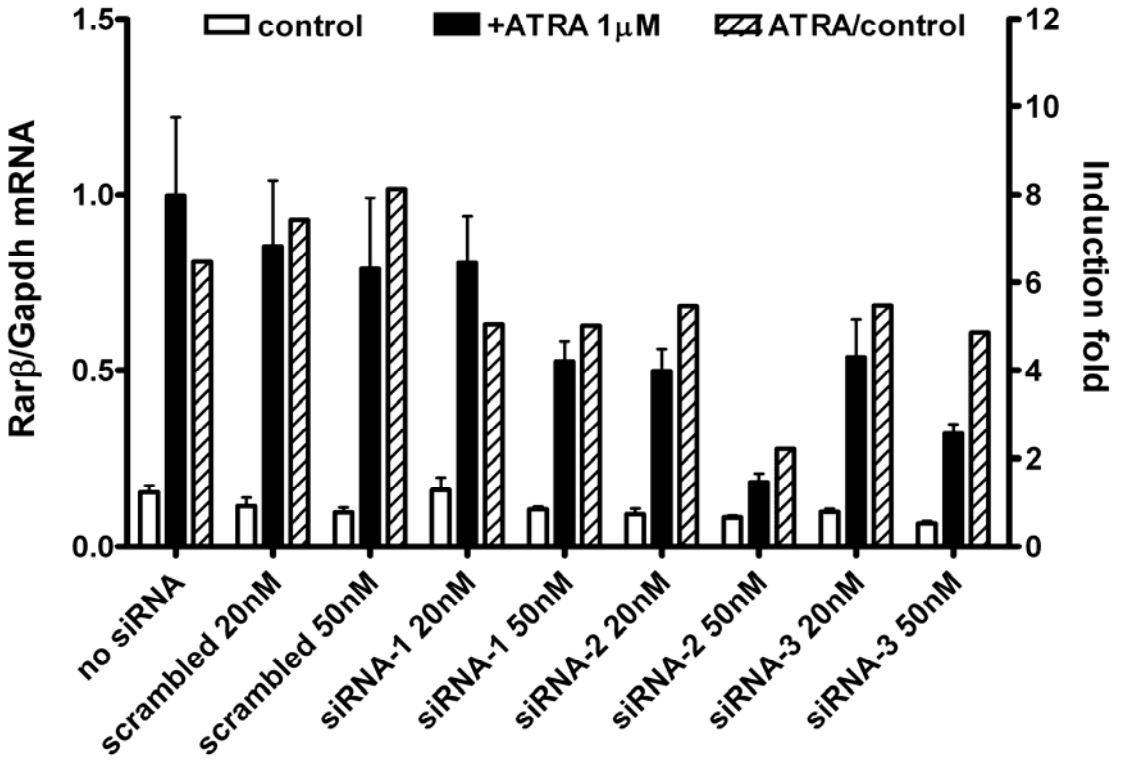
control
+ATRA 1μM
ATRA/control
Rarβ/Gapdh mRNA
Induction fold
1.5
1.0
0.5
0.0
12
10
8
6
4
2
0
no siRNA
scrambled 20nM
scrambled 50nM
siRNA-1 20nM
siRNA-1 50nM
siRNA-2 20nM
siRNA-2 50nM
siRNA-3 20nM
siRNA-3 50nM

Figure 6

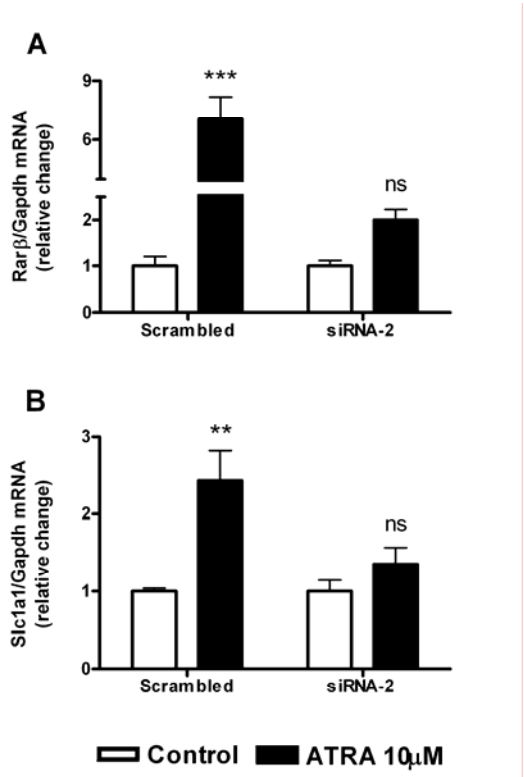
A
Rarβ/Gapdh mRNA (relative change)
9
6
2
1
0
***
ns
Scrambled
siRNA-2
B
Slc1a1/Gapdh mRNA (relative change)
3
2
1
0
**
ns
Scrambled
siRNA-2
Control
ATRA 10μM

Figure 7

Conclusions

Recent experimental evidences in the neuroscience field correlate the alterations in EAAT3/EAAC1 glutamate transporter activity with a number of psychiatric and neurological conditions. Thus, several studies have been carried out to investigate the mechanisms of EAAC1/EAAT3 regulation in various cell models and tissue preparations. Among these, the most studied and well known concern the trafficking and membrane targeting of the intracellular pools of the glutamate transporters, which may be triggered by a number of compounds (Gonzalez et al., 2002; Fournier et al., 2004). These reports strongly suggest the existence of different pools of EAAC1 carriers linked to distinct trafficking pathways.

Until the results presented in this thesis were obtained, no investigation had been carried out on the role of cytoskeleton in these regulations. However, examples exist on the involvement of actin microfilaments in the regulation of other membrane transporters. For example, the integrity of actin cytoskeleton is required for the glutamate-dependent cell surface expression of EAAT1/GLAST transporter in primary glial cell cultures (Duan et al., 1999). Moreover, actin remodelling, mediated by a group of Rho kinases, affects the membrane activity of the Na^+/H^+ exchanger NHE3 in epithelial cell models (Szaszi et al., 2000; Szaszi et al., 2001) and F-actin disruption heavily affects GABA transport by betaine transporter BGT1 (Bricker et al., 2003). In the first contribution here reported we describe that also EAAC1 activity and membrane protein targeting is dependent on the integrity of actin microfilaments. In particular, in C6 cells pre-treated with latrunculin A and cytochalasin D the membrane abundance of EAAC1 was significantly lowered, at least in the absence of phorbols (i.e. in the absence of PKC activation). From these results we should conclude that a fraction of the intracellular EAAC1 population require the integrity the actin cytoskeleton to reach the plasma membrane. However, the increase of EAAC1 activity and membrane abundance triggered by PKC activation were still detectable even after actin disruption, suggesting that PKC-activation may render these “actin-linked pools” of EAAC1 actin-independent. Moreover, a decrease of EAAC1 transport activity were observable also when microtubule toxins were disorganized by colchicine suggesting the existence of EAAC1 pools also trafficked via microtubular pathway. Also in this case the PKC-dependent stimulation of EAAC1 activity was still detectable, suggesting that PKC-sensitive EAAC1 pools are located in external, peripheral locations and represent a kind of fast-moving transporter fraction. .

These results also raise the question of the identity of the molecular partners that connect the transporter with the cytoskeleton. In the second contribution we have started to characterize the relationships between EAAC1 and adducin, an actin binding protein that caps the growing ends of actin filaments and controls its polymerization state (Matsuoka et al., 2000). By co-localization and

co-immunoprecipitation experiments we present evidences pointing to the existence of an interaction between specific intracellular pools of EAAC1 and adducin. The small amount of EAAC1 molecules co-immunoprecipitated with adducin was still detectable after PKC activation. Immunofluorescence experiments indicated the coexistence of EAAC1 and adducin in discrete vesicular bodies of the perinuclear region of highly-expressing C6 cells suggesting that the interaction involves a pool of newly synthesized transporters rather than mature, membrane recycling carriers localized at the cell periphery or in the cell processes. Consistently, although phorbol ester induced an evident marginalization of the transporter, the spots of EAAC1 colocalized with adducin were still detectable.

Other molecular partners of EAAT3/EAAC1 have been already described. Each of them are known to be involved in regulation processes. SNARE family proteins such as SNAP-23 and Syntaxin 1A regulate endocytotic or exocytotic sorting of EAAT3/EAAC1 (Fournier and Robinson, 2006; Yu et al., 2006). More recently, the EAAC1 interacting protein GTRAP3-18, previously described as a modulator of the carrier affinity for the substrate, seems to affect also the ER exit of EAAC1 (Ruggiero et al., 2008). This recent report, in addition with our unpublished results, lead us to propose that adducin may be a novel potential partner of the EAAT3/EAAC1 transporter involved in the sorting of newly synthesized carriers. In particular, the hypothesis that the pools of EAAC1 transporters co-localized with adducin may belong to an early ER pool needs to be evaluated. This will require the characterization of these structures through the identification of compartmental markers and will provide useful insights on the nature of this EAAC1 pool.

In the course of the studies aimed to assess our hypothesis, we used C6 cells transfected with a tagged form of adducin and looked for experimental conditions able to modify the interaction pattern of EAAC1/adducin. We decided to avoid the transfection with EAAC1 constructs because we felt that an excessive and dysregulated expression could lead to the improper regulation of the glutamate transporter. Moreover, it is known that on the -COOH terminal of the protein are present amino acid sequences which are involved in the regulation of the EAAC1 molecular sorting as well as in the modulation of its activity. Therefore, the presence of a carboxy-terminal tag in this region might cause unknown effects on EAAC1 regulation. Thus, we decided to try with differentiating agents, taking advantage of the abundance of stem-like cells in C6 population (Zheng et al., 2007). The differentiation pathways activated in C6 cells could lead to the expression of oligodendrocytic (McMorris, 1983; Lopez-Barahona et al., 1993; Gravel et al., 2000; Pflug et al., 2001), astrocytic (Segovia et al., 1994; Takanaga et al., 2004; Lind et al., 2006) and neuronal markers (Segovia et al., 1994). Among a number of well known differentiating agents, we observed that ATRA induced a strong change in C6 cell morphology and, more interestingly, a huge increase in EAAC1

expression. This approach yielded a strong support to our hypothesis of an adducin-EAAC1 interaction. Moreover, while the effect of ATRA on C6 differentiation has been already studied (Lopez-Barahona et al., 1993), its effect on Slc1a1 gene expression coding for EAAC1 transporter was a completely novel finding. Therefore, in the last contributions here reported we have attempted to characterize the mechanism underlying EAAC1 induced over expression by ATRA. In particular we showed that ATRA leads to the increase of EAAC1 transport activity through an increase of the transporter gene expression as well as of the protein levels. The induction of Slc1a1 gene mRNA by ATRA is a specific phenomenon since neither GLT-1 nor GLAST glutamate transporters are affected by the retinoid.

It is known that ATRA induces the over expression of Plp gene mRNA, a specific oligodendrocytic precursor marker in C6 cells, suggesting that the induction of oligodendrocytic differentiation could be triggered by the treatment. Moreover EAAC1 expression has been described in a sub population of oligodendrocyte precursor cells (OPCs) in *situ* as well as in *vivo* (Domercq et al., 1999; Gottlieb et al., 2000). All together, these results may provide a starting point to study the role of EAAT3/EAAC1 during oligodendrocytes differentiation as well as the physiological mechanisms which trigger its expression. Interestingly, it has been reported that oligodendrocyte are sensitive to excitotoxicity in several pathological conditions (Yoshioka et al., 1996; Pitt et al., 2003), and EAAC1 transporters may be a potential device to reduce the citotoxic effect of the glutamate accumulation. Therefore, it would be interesting to check if EAAC1 overexpression could be detectable also in OPCs or in mature oligodendrocytes after ATRA treatment and evaluate its effects on excitotoxicity under experimental pathological conditions. Preliminary Western Blot experiments, carried out on primary cultures of oligodendrocytes from optic nerve, showed that a slightly increase of EAAC1 protein was detectable after 24 hours of ATRA treatment (unpublished results). However, while this was detected in OPCs from P7 animals, in differentiated cells from P12 animals ATRA did not induce a significant EAAC1 overexpression. Although the phenomenon needs to be confirmed by further experiments including the evaluation of Slc1a1 gene expression before and after ATRA treatment, these preliminary results would correlate well with those obtained in C6 cells.

In the last contribution included in this thesis we have begun the characterization of ATRA effect on Slc1a1 gene over expression. Through a pharmacological approach, we found evidences indicating a predominant role for a RAR in the stimulation of glutamate transport induced by ATRA. The molecular mechanism underlying the ATRA-dependent Slc1a1 gene mRNA induction has been also provisionally elucidated. In particular, we observed that the retinoid did not affect the Slc1a1 mRNA half-life and that the gene induction required the new synthesis of a protein

intermediate. It is known that in C6 cell line as well as in other cell models ATRA treatment increased both Rarb mRNA (Lopez-Barahona et al., 1993) and protein levels (De los Santos et al., 2007). As expected, also under our experimental conditions the increase of both Rarb mRNA and protein were detectable at earlier times of ATRA treatment. This result suggests that Slc1a1 is a RARbeta-target gene. Consistently, Rarb silencing substantially prevents the ATRA-dependent induction of Slc1a1 gene mRNA. However a direct involvement of RAR beta in the Slc1a1 transcription needs to be investigated at the gene level. Possible useful approaches to study more in depth this phenomenon could be the use of cell models transfected with dominant negative forms of Rarb or to perform experiments of chromatin immunoprecipitation (ChiP) for the research of RARE sequences in the Slc1a1 gene. The elucidation of these molecular mechanisms will represent a step toward a possible pharmacological control of EAAC1 expression and, hence, of the uptake of excitatory amino acids in the CNS.

References

Barkalow KL, Italiano JE, Jr., Chou DE, Matsuoka Y, Bennett V, Hartwig JH (2003) Alpha-adducin dissociates from F-actin and spectrin during platelet activation. J Cell Biol 161:557-570.

Bensimon G, Lacomblez L, Meininger V (1994) A controlled trial of riluzole in amyotrophic lateral sclerosis. ALS/Riluzole Study Group. N Engl J Med 330:585-591.

Bianhi MG, Gatti R, Torielli L, Pdoani G, Gazzola GC, Bussolati O (2009) EAAC1 glutamate transporter interacts with the actin-binding protein α-adducin in c6 glioma cells. Neuroscience submitted.

Bianchi MG, Cagnin S, Kagechika H, Gazzola GC, Bussolati O (2009) The ATRA-dependent overexpression of the glutamate transporter EAAC1 requires RARβ induction. Biochim Biophys Acta submitted.

Bianchi MG, Gazzola GC, Tognazzi L, Bussolati O (2008) C6 glioma cells differentiated by retinoic acid overexpress the glutamate transporter excitatory amino acid carrier 1 (EAAC1). Neuroscience 151:1042-1052.

Bianchi MG, Rotoli BM, Dall'asta V, Gazzola GC, Gatti R, Bussolati O (2006) PKC-dependent stimulation of EAAT3 glutamate transporter does not require the integrity of actin cytoskeleton. Neurochem Int 48:341-349.

Bortolotto ZA, Fitzjohn SM, Collingridge GL (1999) Roles of metabotropic glutamate receptors in LTP and LTD in the hippocampus. Curr Opin Neurobiol 9:299-304.

Bricker JL, Chu S, Kempson SA (2003) Disruption of F-actin stimulates hypertonic activation of the BGT1 transporter in MDCK cells. Am J Physiol Renal Physiol 284:F930-937.

Choi DW (1996) Ischemia-induced neuronal apoptosis. Curr Opin Neurobiol 6:667-672.

Crino PB, Jin H, Shumate MD, Robinson MB, Coulter DA, Brooks-Kayal AR (2002) Increased expression of the neuronal glutamate transporter (EAAT3/EAAC1) in hippocampal and neocortical epilepsy. Epilepsia 43:211-218.

Dall'Asta V, Gazzola GC, Franchi-Gazzola R, Bussolati O, Longo N, Guidotti GG (1983) Pathways of L-glutamic acid transport in cultured human fibroblasts. J Biol Chem 258:6371-6379.

Danbolt NC (2001) Glutamate uptake. Prog Neurobiol 65:1-105.

Davis KE, Straff DJ, Weinstein EA, Bannerman PG, Correale DM, Rothstein JD, Robinson MB (1998) Multiple signaling pathways regulate cell surface expression and activity of the excitatory amino acid carrier 1 subtype of Glu transporter in C6 glioma. J Neurosci 18:2475-2485.

De los Santos M, Zambrano A, Sanchez-Pacheco A, Aranda A (2007) Histone deacetylase inhibitors regulate retinoic acid receptor beta expression in neuroblastoma cells by both transcriptional and posttranscriptional mechanisms. Mol Endocrinol 21:2416-2426.

Dinsmore J, Ratliff J, Deacon T, Pakzaban P, Jacoby D, Galpern W, Isacson O (1996) Embryonic stem cells differentiated in vitro as a novel source of cells for transplantation. Cell Transplant 5:131-143.

Domercq M, Sanchez-Gomez MV, Areso P, Matute C (1999) Expression of glutamate transporters in rat optic nerve oligodendrocytes. Eur J Neurosci 11:2226-2236.

Duan S, Anderson CM, Stein BA, Swanson RA (1999) Glutamate induces rapid upregulation of astrocyte glutamate transport and cell-surface expression of GLAST. J Neurosci 19:10193-10200.

Dunlop J, Beal McIlvain H, She Y, Howland DS (2003) Impaired spinal cord glutamate transport capacity and reduced sensitivity to riluzole in a transgenic superoxide dismutase mutant rat model of amyotrophic lateral sclerosis. J Neurosci 23:1688-1696.

Efendiev R, Krmar RT, Ogimoto G, Zwiller J, Tripodi G, Katz AI, Bianchi G, Pedemonte CH, Bertorello AM (2004) Hypertension-linked mutation in the adducin alpha-subunit leads to higher AP2-mu2 phosphorylation and impaired Na+,K+-ATPase trafficking in response to GPCR signals and intracellular sodium. Circ Res 95:1100-1108.

Fournier KM, Gonzalez MI, Robinson MB (2004) Rapid trafficking of the neuronal glutamate transporter, EAAC1: evidence for distinct trafficking pathways differentially regulated by protein kinase C and platelet-derived growth factor. J Biol Chem 279:34505-34513.

Fournier KM, Robinson MB (2006) A dominant-negative variant of SNAP-23 decreases the cell surface expression of the neuronal glutamate transporter EAAC1 by slowing constitutive delivery. Neurochem Int 48:596-603.

Franchi-Gazzola R, Visigalli R, Bussolati O, Gazzola GC (1996) Involvement of protein kinase Cepsilon in the stimulation of anionic amino acid transport in cultured human fibroblasts. J Biol Chem 271:26124-26130.

Furuta A, Noda M, Suzuki SO, Goto Y, Kanahori Y, Rothstein JD, Iwaki T (2003) Translocation of glutamate transporter subtype excitatory amino acid carrier 1 protein in kainic acid-induced rat epilepsy. Am J Pathol 163:779-787.

Gazzola GC, Dall'Asta V, Bussolati O, Makowske M, Christensen HN (1981) A stereoselective anomaly in dicarboxylic amino acid transport. J Biol Chem 256:6054-6059.

Gilligan DM, Sarid R, Weese J (2002) Adducin in platelets: activation-induced phosphorylation by PKC and proteolysis by calpain. Blood 99:2418-2426.

Gonzalez MI, Bannerman PG, Robinson MB (2003) Phorbol myristate acetate-dependent interaction of protein kinase Calpha and the neuronal glutamate transporter EAAC1. J Neurosci 23:5589-5593.

Gonzalez MI, Kazanietz MG, Robinson MB (2002) Regulation of the neuronal glutamate transporter excitatory amino acid carrier-1 (EAAC1) by different protein kinase C subtypes. Mol Pharmacol 62:901-910.

Gonzalez MI, Susarla BT, Fournier KM, Sheldon AL, Robinson MB (2007) Constitutive endocytosis and recycling of the neuronal glutamate transporter, excitatory amino acid carrier 1. J Neurochem 103:1917-1931.

Gonzalez MI, Robinson MB (2004) Protein kinase C-dependent remodeling of glutamate transporter function. Mol Interv 4:48-58.

Gottlieb M, Domercq M, Matute C (2000) Altered expression of the glutamate transporter EAAC1 in neurons and immature oligodendrocytes after transient forebrain ischemia. J Cereb Blood Flow Metab 20:678-687.

Gravel M, Gao E, Hervouet-Zeiber C, Parsons V, Braun PE (2000) Transcriptional regulation of 2',3'-cyclic nucleotide 3'-phosphodiesterase gene expression by cyclic AMP in C6 cells. J Neurochem 75:1940-1950.

Han F, Shioda N, Moriguchi S, Qin ZH, Fukunaga K (2008) Downregulation of glutamate transporters is associated with elevation in extracellular glutamate concentration following rat microsphere embolism. Neurosci Lett 430:275-280.

Hinoi E, Takarada T, Ueshima T, Tsuchihashi Y, Yoneda Y (2004) Glutamate signaling in peripheral tissues. Eur J Biochem 271:1-13.

Jang YK, Park JJ, Lee MC, Yoon BH, Yang YS, Yang SE, Kim SU (2004) Retinoic acid-mediated induction of neurons and glial cells from human umbilical cord-derived hematopoietic stem cells. J Neurosci Res 75:573-584.

Krizman-Genda E, Gonzalez MI, Zelenaia O, Robinson MB (2005) Evidence that Akt mediates platelet-derived growth factor-dependent increases in activity and surface expression of the neuronal glutamate transporter, EAAC1. Neuropharmacology 49:872-882.

Lecuona E, Ridge K, Pesce L, Batlle D, Sznajder JI (2003) The GTP-binding protein RhoA mediates Na,K-ATPase exocytosis in alveolar epithelial cells. Mol Biol Cell 14:3888-3897.

Lin CI, Orlov I, Ruggiero AM, Dykes-Hoberg M, Lee A, Jackson M, Rothstein JD (2001) Modulation of the neuronal glutamate transporter EAAC1 by the interacting protein GTRAP3-18. Nature 410:84-88.

Lind CR, Gray CW, Pearson AG, Cameron RE, O'Carroll SJ, Narayan PJ, Lim J, Dragunow M (2006) The mitogen-activated/extracellular signal-regulated kinase kinase 1/2 inhibitor U0126 induces glial fibrillary acidic protein expression and reduces the proliferation and migration of C6 glioma cells. Neuroscience 141:1925-1933.

Lopez-Barahona M, Minano M, Mira E, Iglesias T, Stunnenberg HG, Rodriguez-Pena A, Bernal J, Munoz A (1993) Retinoic acid posttranscriptionally up-regulates proteolipid protein gene expression in C6 glioma cells. J Biol Chem 268:25617-25623.

Lortet S, Canolle B, Masmejean F, Nieoullon A (2008) Plasma membrane expression of the neuronal glutamate transporter EAAC1 is regulated by glial factors: evidence for different regulatory pathways associated with neuronal maturation. Neurochem Int 52:1373-1382.

Luhmann HJ (1996) Ischemia and lesion induced imbalances in cortical function. Prog Neurobiol 48:131-166.

Ma K, Zheng S, Zuo Z (2006) The transcription factor regulatory factor X1 increases the expression of neuronal glutamate transporter type 3. J Biol Chem 281:21250-21255.

Maden M (2007) Retinoic acid in the development, regeneration and maintenance of the nervous system. Nat Rev Neurosci 8:755-765.

Matsuoka Y, Li X, Bennett V (1998) Adducin is an in vivo substrate for protein kinase C: phosphorylation in the MARCKS-related domain inhibits activity in promoting spectrin-actin complexes and occurs in many cells, including dendritic spines of neurons. J Cell Biol 142:485-497.

Matsuoka Y, Li X, Bennett V (2000) Adducin: structure, function and regulation. Cell Mol Life Sci 57:884-895.

McMorris FA (1983) Two independent mechanisms of induction of 2':3'-cyclic-nucleotide 3'-phosphohydrolase in glioma cells by cyclic AMP and high cell density. Biochim Biophys Acta 762:560-568.

Meador-Woodruff JH, Clinton SM, Beneyto M, McCullumsmith RE (2003) Molecular abnormalities of the glutamate synapse in the thalamus in schizophrenia. Ann N Y Acad Sci 1003:75-93.

Molinuevo JL, Llado A, Rami L (2005) Memantine: targeting glutamate excitotoxicity in Alzheimer's disease and other dementias. Am J Alzheimers Dis Other Demen 20:77-85.

Nieoullon A, Canolle B, Masmejean F, Guillet B, Pisano P, Lortet S (2006) The neuronal excitatory amino acid transporter EAAC1/EAAT3: does it represent a major actor at the brain excitatory synapse? J Neurochem 98:1007-1018.

Pflug BR, Colangelo AM, Tornatore C, Mocchetti I (2001) TrkA induces differentiation but not apoptosis in C6-2B glioma cells. J Neurosci Res 64:636-645.

Pitt D, Nagelmeier IE, Wilson HC, Raine CS (2003) Glutamate uptake by oligodendrocytes: Implications for excitotoxicity in multiple sclerosis. Neurology 61:1113-1120.

Plaitakis A, Shashidharan P (2000) Glutamate transport and metabolism in dopaminergic neurons of substantia nigra: implications for the pathogenesis of Parkinson's disease. J Neurol 247 Suppl 2:II25-35.

Proper EA, Hoogland G, Kappen SM, Jansen GH, Rensen MG, Schrama LH, van Veelen CW, van Rijen PC, van Nieuwenhuizen O, Gispen WH, de Graan PN (2002) Distribution of glutamate transporters in the hippocampus of patients with pharmaco-resistant temporal lobe epilepsy. Brain 125:32-43.

Rakhade SN, Loeb JA (2008) Focal reduction of neuronal glutamate transporters in human neocortical epilepsy. Epilepsia 49:226-236.

Raman C, McAllister SD, Rizvi G, Patel SG, Moore DH, Abood ME (2004) Amyotrophic lateral sclerosis: delayed disease progression in mice by treatment with a cannabinoid. Amyotroph Lateral Scler Other Motor Neuron Disord 5:33-39.

Rothstein JD, Dykes-Hoberg M, Pardo CA, Bristol LA, Jin L, Kuncl RW, Kanai Y, Hediger MA, Wang Y, Schielke JP, Welty DF (1996) Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate. Neuron 16:675-686.

Ruggiero AM, Liu Y, Vidensky S, Maier S, Jung E, Farhan H, Robinson MB, Sitte HH, Rothstein JD (2008) The endoplasmic reticulum exit of glutamate transporter is regulated by the inducible mammalian Yip6b/GTRAP3-18 protein. J Biol Chem 283:6175-6183.

Sanchez-Ramos JR, Song S, Kamath SG, Zigova T, Willing A, Cardozo-Pelaez F, Stedeford T, Chopp M, Sanberg PR (2001) Expression of neural markers in human umbilical cord blood. Exp Neurol 171:109-115.

Segovia J, Lawless GM, Tillakaratne NJ, Brenner M, Tobin AJ (1994) Cyclic AMP decreases the expression of a neuronal marker (GAD67) and increases the expression of an astroglial marker (GFAP) in C6 cells. J Neurochem 63:1218-1225.

Sen I, Nalini A, Joshi NB, Joshi PG (2005) Cerebrospinal fluid from amyotrophic lateral sclerosis patients preferentially elevates intracellular calcium and toxicity in motor neurons via AMPA/kainate receptor. J Neurol Sci 235:45-54.

Sepkuty JP, Cohen AS, Eccles C, Rafiq A, Behar K, Ganel R, Coulter DA, Rothstein JD (2002) A neuronal glutamate transporter contributes to neurotransmitter GABA synthesis and epilepsy. J Neurosci 22:6372-6379.

Sheldon AL, Gonzalez MI, Robinson MB (2006) A carboxyl-terminal determinant of the neuronal glutamate transporter, EAAC1, is required for platelet-derived growth factor-dependent trafficking. J Biol Chem 281:4876-4886.

Szaszi K, Kurashima K, Kaibuchi K, Grinstein S, Orlowski J (2001) Role of the cytoskeleton in mediating cAMP-dependent protein kinase inhibition of the epithelial Na+/H+ exchanger NHE3. J Biol Chem 276:40761-40768.

Szaszi K, Kurashima K, Kapus A, Paulsen A, Kaibuchi K, Grinstein S, Orlowski J (2000) RhoA and rho kinase regulate the epithelial Na+/H+ exchanger NHE3. Role of myosin light chain phosphorylation. J Biol Chem 275:28599-28606.

Takanaga H, Yoshitake T, Hara S, Yamasaki C, Kunimoto M (2004) cAMP-induced astrocytic differentiation of C6 glioma cells is mediated by autocrine interleukin-6. J Biol Chem 279:15441-15447.

Torielli L, Tivodar S, Montella RC, Iacone R, Padoani G, Tarsini P, Russo O, Sarnataro D, Strazzullo P, Ferrari P, Bianchi G, Zurzolo C (2008) alpha-Adducin mutations increase Na/K pump activity in renal cells by affecting constitutive endocytosis: implications for tubular Na reabsorption. Am J Physiol Renal Physiol 295:F478-487.

Tripodi G, Valtorta F, Torielli L, Chieregatti E, Salardi S, Trusolino L, Menegon A, Ferrari P, Marchisio PC, Bianchi G (1996) Hypertension-associated point mutations in the adducin alpha and beta subunits affect actin cytoskeleton and ion transport. J Clin Invest 97:2815-2822.

Yang Y, Kinney GA, Spain WJ, Breitner JC, Cook DG (2004) Presenilin-1 and intracellular calcium stores regulate neuronal glutamate uptake. J Neurochem 88:1361-1372.

Yoshioka A, Bacskai B, Pleasure D (1996) Pathophysiology of oligodendroglial excitotoxicity. J Neurosci Res 46:427-437.

Yu YX, Shen L, Xia P, Tang YW, Bao L, Pei G (2006) Syntaxin 1A promotes the endocytic sorting of EAAC1 leading to inhibition of glutamate transport. J Cell Sci 119:3776-3787.

Zheng X, Shen G, Yang X, Liu W (2007) Most C6 cells are cancer stem cells: evidence from clonal and population analyses. Cancer Res 67:3691-3697.