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Activity dependent internalization of the glutamate transporter GLT-1 mediated by β-arrestin 1 and ubiquitination

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ABSTRACT

GLT-1 is the main glutamate transporter in the brain and undergoes trafficking processes that control its concentration on the cell surface thereby shaping glutamatergic neurotransmission. We have investigated how the traffic of GLT-1 is regulated by transporter activity. We report that internalization of GLT-1 from the cell surface is accelerated by transportable substrates like glutamate or aspartate, as well as by the transportable inhibitor L-trans-2,4-PDC, but not by the non-substrate inhibitor WAY 213613 in primary mixed cultures and in transiently transfected HEK293 cells. Analysis of the mechanism of endocytosis in HEK293 cells revealed that glutamate promoted the association with the transporter of the adaptor protein β-arrestin and the ubiquitin ligase Nedd4-2. The addition of glutamate is accompanied by an increase in the transporter ubiquitination, and the internalization is suppressed by an ubiquitination inhibitor (PYR41), and in a mutant defective in C-terminal lysines. The glutamate triggered endocytosis was also suppressed by siRNA for β-arrestin. This regulatory mechanism might be relevant in controlling the amount of transporter on the cell surface in conditions such as ischemia or traumatic brain injury, where extracellular concentrations of glutamate are persistently elevated.

1. Introduction

Synaptic transmission relies on the efficient removal of neurotransmitters from the synaptic cleft. For glutamate, the main excitatory neurotransmitter in the brain, this task is performed by specific high affinity glutamate transporters of the glutamate/neutral amino acid transporters (SLC1) family. SLC1 includes five glutamate transporters called EAAT1/GLAST, EAAT2/GLT-1, EAAT3/EAAC1, EAAT4 and EAAT5. One of these, GLT-1, is responsible for up to 90% of the clearance of extracellular glutamate in the forebrain and is located mainly in the glial cells, although neuronal forms of the transporter also exist (Tanaka et al., 1997; Petr et al., 2015). GLT-1 not only contributes to physiological glutamatergic neurotransmission but is also responsible for maintaining glutamate to below excitotoxic levels, the regulation of which is critical in pathological conditions such as ischemia, or traumatic brain injury (Lai et al., 2014). Like many other membrane transporters, the trafficking of GLT-1 protein to and from the plasma membrane provides a means of rapidly regulating its activity (González and Robinson, 2004; Robinson, 2006). In astrocytes, the major isoform of GLT-1, GLT1a, was shown to reside transiently at the plasma membrane and traffics rapidly to and from the cell surface, establishing a dynamic, constitutively cycling pool of carriers with the potential for rapid mobilization. By contrast, the minor isoform of GLT-1, GLT1b, is more stably anchored to the membrane via interaction with proteins containing PDZ domains, and is mobilized by increases in intracellular calcium and activation of CaMKII (Underhill et al., 2015). Other intracellular signaling events are also known to regulate these trafficking events, for instance, PKC-triggered internalization of GLT-1 has been demonstrated both in heterologous and in endogenous systems, and probably involves the operation of a variety of endocytic mechanisms that target the transporter into recycling endosomes and lysosomes (Cremona et al., 2011). Ubiquitination has been shown to be one of these mechanisms as both GLT-1 became internalized in response to phorbol esters in a process dependent on the ubiquitin ligase Nedd4-2, an E3 ubiquitin ligase of the HECT family (Sheldon et al., 2008; González-González

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et al., 2008; García-Tardón et al., 2012). Recent studies indicate that GLT-1 is clustered in astrocytic processes and that its distribution on the cell surface is regulated by neuronal activity over a time-scale of minutes (Benediktsson et al., 2012). These clusters are mobilized and dissociated by glutamate released in its neighbourhood (Murphy-Royal et al., 2015). Although some studies have suggested that glutamate acting through glutamate receptors might regulate transport activity (Otis et al., 2014), a mechanism that remains unexplored is the possibility that glutamate binding and/or its transport itself might trigger endocytosis thereby modulating the concentration of GLT-1 on the cell surface. Indeed, several neurotransmitter transporters such as the dopamine or the serotonin transporters have been shown to traffic to or from the plasma membrane in the presence of specific ligands, although the molecular mechanisms of these events remain poorly defined (Saunders et al., 2000; Ramamoorthy and Blakely, 1999). These mechanisms might be especially relevant for glutamate since its concentration oscillates in the extracellular milieu of the nervous system from the micromolar range during basal activity to around 1 mM after neuronal firing (Clements et al., 1992). These levels can be even higher in ischemic episodes or in traumatic brain injury, pathological situations where the concentration of glutamate is tonically raised for several hours or even days (Nishizawa, 2001). GLT-1 is thought to play a central role in the response of brain to fluctuations in the glutamate levels (Mitani and Tanaka, 2003).

2. Material and methods

2.1. Materials

EZ-Link Sulfo-NHS-SS-Biotin was from Pierce. Protein standards for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (NZYColour Protein Marker II) were from NZYtech. Lipofectamine™ 2000, foetal calf serum and pcDNA3 plasmid were purchased from Invitrogen; TrueFect-Lipo™ was from United Biosystems (Rockville, MD); and phenylmethanesulfonyl fluoride (PMSF), the Expand High Fidelity PCR system, and all restriction enzymes were obtained from Roche Applied Science or New England Biolabs. The TNT® T7 Quick Coupled Transcription/Translation System was from Promega and the [^{35}S]-Methionine (1175 Ci/mmol, 10,2 mCi/ml) was from PerkinElmer. The QuikChange Site-Directed Mutagenesis kit was from Agilent; nitrocellulose sheets and Clarity™ Western Blot ECL Substrate were from Bio-Rad. The monoclonal mouse anti-hemagglutinin (HA) (clone 12CA5) was prepared at the microscopy service of the Centro de Biología Molecular (Madrid, Spain); polyclonal anti-GLT-1 was from Merck-Millipore (ref. AB1783), monoclonal anti-Nedd4-2 was from Bethyl Laboratories (Montgomery, TX) (ref. A302-513A), anti-β-arrestin 1 was from BD Bioscience (San Diego, CA) (ref. 610550), the Alexa Fluor 488-coupled goat anti-mouse secondary antibodies were obtained from Molecular Probes (Eugene, OR), and the mouse monoclonal anti-ubiquitin (P4D1) was from Santa Cruz Biotechnology (Santa Cruz, CA). The oligonucleotides used were synthesized by Sigma-Aldrich. All other chemicals were obtained also from Sigma-Aldrich.

2.2. Plasmid constructs

The various HA-tagged GLT-1 and mutants were prepared as previously described (García-Tardón et al., 2012). Truncated forms of Nedd4-2 were produced by PCR and cloned into pGEX 5X-3 using as a template the mouse Nedd4-2. The sequence specific oligonucleotides contained restriction sites for Sal I (forward) and Not I (reverse) and were designed to include the following residues (enumeration refers to mouse Nedd4-2, isoform 2): GST-1 (1-855), GST-2 (520-855), GST-3 (1-507), GST-4 (1-420), GST-5 (1-376), GST-6 (1-308), GST-7 (1-105), GST-8 (106-308), GST-9 (1-855, mutated W405Y/P408A). All constructs and mutants were confirmed by sequencing.

2.3. Primary cortical neurons

Primary cortical neurons were prepared from cortices of embryonic day 17–18 (E17-18) Wistar rat feotuses as described by Brewer (1995) with modifications. The cortex was dissected and individual cells mechanically disaggregated in HBSS (Invitrogen) containing 0.25% trypsin (Invitrogen) and 4 mg/ml DNase (Sigma). Cells were plated at a density of 1.5×10^6 per 6 cm culture plate (Falcon), in plates pretreated with poly-D-Lysine, and incubated for 4 h in DMEM containing 10% FCS, 10 mM glucose, 10 mM sodium pyruvate, 0.5 mM glutamine, 0.05 mg/ml gentamicin, 0.01% streptomycin, and 100 μU/ml penicillin G. Finally, this medium was replaced with Neurobasal/B27 culture medium containing 0.5 mM glutamine. Cytosine arabinoside was not included in the culture medium to allow glial proliferation.

2.4. Cell growth and transfection

HEK293 and MDCK cells (American Type Culture Collection) were grown in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37 °C in an atmosphere of 5% CO_2. Transient expression in HEK293 or MDCK cells was achieved using TrueFect-Lipo or Lipofectamine 2000, respectively, according to the manufacturers' instructions. The cells were incubated for 48 h at 37 °C and then analyzed biochemically or by immunofluorescence.

2.5. Cell surface biotinylation

Cell surface biotinylation with the non-permeable Sulfo–NHS–SS-Biotin reagent in transfected HEK293 and primary cultures, isolation of biotinylated proteins and analysis by western blots was performed as described previously (Jiménez et al., 2011).

2.6. Electrophoresis and immunoblotting

SDS-PAGE was performed on 7.5% polyacrylamide gels in the presence of 2-mercaptoethanol. After electrophoresis, the protein samples were transferred to nitrocellulose membranes in a semidry electroblotting system at 1.2 mA/cm2 for 2 h (LKB) using a transfer buffer containing 192 mM glycine and 25 mM Tris-HCl (pH 8.3). Nonspecific binding to the membrane was blocked by incubating the filter with 5% nonfat milk protein in 10 mM Tris-HCl (pH 7.5), 150 mM NaCl for 4 h at 25 °C. The membrane was then probed overnight at 4 °C with the diluted primary antibody (anti-HA or anti-ubiquitin), which was detected after washing with an anti-mouse IgG peroxidase-linked secondary antibody. The labeled bands were visualized by ECL and quantified by densitometry on a GS-710 calibrated imaging densitometer from Bio-Rad with Quantity One software by using film exposures in the linear range. For GLT-1, most of the protein appeared as oligomers, and these were the bands used for quantification. For proteins labeled with ^{32}P, the dried gels were rehydrated in 50 mM ammonium bicarbonate for 16 h, and the proteins were transferred to polyvinylidene fluoride (PVDF) membranes before they were incubated with the primary and secondary antibodies.

2.7. Immunoprecipitation

Immunoprecipitation was performed as described in (García-Tardón et al., 2012).