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Differential regulation of the glutamate transporters GLT-1 and GLAST by GSK3β

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ABSTRACT

The glutamate transporters GLAST and GLT-1 are mainly expressed in glial cells and regulate glutamate levels in the synapses. GLAST and GLT-1 are the targets of several signaling pathways. In this study we explore the possible functional interaction between these transporters and GSK3β. This kinase is involved in multiple cellular processes including neuronal development and synaptic plasticity. To evaluate whether GLT-1 and GLAST were regulated by GSK3β, we coexpresed these proteins in heterologous expression systems. In both COS-7 cells and *Xenopus laevis* oocytes, GSK3β stimulated the activity of GLT-1 and reduced that of GLAST. These effects were associated with corresponding changes in the amounts of GLT-1 or GLAST in the plasma membrane. These effects were suppressed by inhibitors of GSK3β or a catalytically inactive form of the kinase. GSK3β also decreases the incorporation of ^{32}Pi into GLT-1 and increases GLAST phosphorylation. Pharmacological inhibition of endogenous GSK3β in primary cultures of rat brain cortex also leads to a differential modulation of GLT-1 and GLAST. Our results suggest that constitutively active GSK3β is important in controlling the expression of functional glutamate transporters on the plasma membrane. This regulation might be relevant in physiological and pathological conditions in which glutamate transporters and GSK3β signaling are involved.

1. Introduction

Glutamate is the major mediator of excitatory signals in the mammalian central nervous system. It is involved in most aspects of normal brain function including cognition, memory and learning. It also plays a major role in the control of the synapse plasticity, cell migration, differentiation and death. Glutamate exerts its signaling role by acting on glutamate receptors. Excessive activation of glutamate receptors causes cell death (Choi et al., 1987). The clearance of extracellular glutamate is accomplished by five glutamate transporters from the *SLC1* gene family: EAAC1 / EAAT3 (Kanai and Hediger, 1992), GLT-1 / EAAT2 (Pines et al., 1992), GLAST / EAAT1 (Storck et al., 1992; Tanaka, 1993), EAAT4 (Fairman et al., 1995) and EAAT5 (Arriza et al., 1997). GLT-1 and GLAST are mainly glial proteins, while EAAT3, EAAT4 and EAAT5 are neuronal (Danbolt, 2001).

Although GLAST and GLT-1 are found predominantly in glial cells, they display specific regional and developmental patterns of expression (Bar-Peled et al., 1997; Furuta et al., 1997; Lehre et al., 1995; Schmitt et al., 1996, 1997; Shibata et al., 1996; Sutherland et al., 1996; Ullensvang et al., 1997; Chen et al., 2002; Chen et al., 2004). GLAST is more abundant in the cerebellum than in forebrain or brainstem, whereas GLT-1 has a more uniform expression pattern (Lehre et al., 1995). Generally, in rodent brain the expression of GLAST precedes that of GLT-1 during postnatal development, although both increase stepwise, especially during the period of synaptogenesis, reaching adult values, or even higher, by the third and fourth postnatal week (Furuta et al., 1997; Shibata et al., 1996; Sutherland et al., 1996). The expression of GLT-1 protein (but not GLAST) is very prominent in some white matter tracts in fetal rat CNS (Furuta et al., 1997). With regard to cellular localization, GLAST expression is high in specialized glial cells such as the Bergmann glia of the cerebellum, the Müller cells of the retina and supporting glia in the vestibular end

Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; CNS, central nervous system; COS-7, cells CV-1 (simian) in Origin and carrying the SV40 genetic material; DIV, days *in vitro*; DTT, dithiothreitol; EAAC1, excitatory amino-acid carrier 1; EAAT, excitatory amino-acid transporter; EGTA, ethyleneglycoltetraacetic acid; EDTA, ethylenediaminetetraacetic acid; GLT-1, glutamate transporter-1; GLAST, glutamate aspartate transporter; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PI3K, phosphoinositide 3-kinase; PKA, protein kinase A; PKC, protein kinase C; PSD-95, postsynaptic density protein 95; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SLC1, solute carrier 1; TDZD-8, 4-Benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione.

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organ (Danbolt, 2001), where it performs specific functions. For instance, retinal GLAST is required for normal signal transmission between photoreceptors and bipolar cells, and both GLAST and GLT-1 play a neuroprotective role during ischemia (Harada et al., 1998). The cellular distribution of GLT-1, especially the GLT-1b variant has been a matter of controversy. Quantitative measures by Danbolt and colleagues confirm that most of the transporter, about 80% is located in astrocytes, but there is a significative amount of GLT-1 in neuronal structures (Furness et al., 2008). Down-regulation of GLT-1 is suspected to be linked to neurodegenerative diseases such as Alzheimer's disease, Huntington's disease and amyotrophic lateral sclerosis (Cassano et al., 2012; Miller et al., 2008; Trotti et al., 2001).

Glycogen synthase kinase 3 (GSK3) is a serine-threonine kinase involved in many cellular processes such as cell proliferation and differentiation (Frame and Cohen, 2001), cell survival (Takashima et al., 1993) and cell motility (Lucas et al., 1998; Sanchez et al., 2001; Wagner et al., 1997). There are two isoforms of GSK3 kinase, α and β, and these are encoded by two different genes. The two isoforms have extensive homology and similar functions. GSK3β, particularly the GSK3β2 variant, predominates in the CNS (Mukai et al., 2002). GSK3β is a key regulator in many neurodevelopmental processes including neurogenesis, neuronal migration, polarization and axon growth and guidance. GSK3β has been associated with many neurodegenerative and psychiatric diseases such as Alzheimer's disease, schizophrenia, bipolar syndrome and autism, and it is a potential therapeutic target for psychiatric drugs (Bhat et al., 2004).

The involvement of GSK3β in the modulation of glutamatergic transmission via NMDA and AMPA receptors prompted us to investigate the effect of this kinase on the activity of the glutamate transporters GLAST and GLT-1. Our results provide evidence of a differential regulation of GLT-1 and GLAST by GSK3β.

2. Materials and methods

2.1. Materials

L-[^{3}H]Glutamate (PerkinElmer), protein standards for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (Precision Plus Standards) (Bio-Rad), and the Enhanced Chemiluminescence (ECL) Western blotting detection reagents were obtained from Amersham. pcDNA3 plasmid was purchased from Invitrogen; TrueFect-LipoTM 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. The QuikChange Site-Directed Mutagenesis kit was from Stratagene (La Jolla, CA), nitrocellulose sheets were from Bio-Rad, and foetal calf serum was supplied by Invitrogen. Dulbecco's Modified Eagle medium, 0.25% Tripsin, Neurobasal and B27 were from Invitrogen. The polyclonal rat anti-GLT-1 was provided by Chemicon and anti-GLAST was provided by Abcam. The anti-GAPDH and anti-NaK^+ ATPase were provided by Cell Signaling Technology and Santa Cruz Biotechnology respectively. The monoclonal mouse anti-hemagglutinin (HA) (clone 12CA5) was prepared at the microscopy service of the Centro de Biología Molecular (Madrid, Spain). The goat anti-rat and goat anti-mouse secondary antibodies were obtained from Molecular Probes (Eugene, OR). EZ-Link Sulfo-NHS-SSBiotin was from Pierce. The pGEM-T easy cloning vector was purchased from Promega (Madison, WI), and the oligonucleotides used were synthesized by Sigma. All other chemicals were obtained from Sigma.

Wistar rats were bred at the Centro de Biología Molecular Severo Ochoa (Madrid, Spain). The experiments were performed in accordance with the Royal Decree 1201/2005 of the Spanish Ministry of Presidency for the protection of animals used in scientific research.

2.2. Cell growth and transfection

COS-7 cells (American Type Culture Collection) were grown in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum at 37 °C in an atmosphere of 95% Air/ 5% CO_2. Transient expression in COS-7 cells was achieved using TrueFect-Lipo, according to the manufacturers' instructions. The cells were incubated for 48 h at 37 °C and then analyzed biochemically or by immunofluorescence and/or in transport assays.

2.3. Brain cortex primary cultures

Brain cortex primary cultures were performed as described (Kaech and Banker, 2007) with modifications. Brain cortex from 18-day-old rat foetuses were isolated in Hanks balanced salt solution (Invitrogen) and dissociated with 0.25% Trypsin and 4 mg/ml DNAase. Cells were incubated for 4 h in plating buffer (Dulbecco's Modified Eagle Medium; containing 10% foetal calf serum and supplemented with 10 mM glucose, 10 mM sodium pyruvate, 0.5 mM glutamine, 0.05 mg/ml gentamicin, 0.01% streptomycin, 100 μU / ml penicillin G), and buffer was then replaced by culture medium (Neurobasal/B27 50:1 by vol, containing 0.5 mM glutamine. Cells were plated on D-poly-lysine (13 μg/ml)-coated 24-well-plates at a density of 200000 cells/well for transport assays or 6-well-plates at a density of 800000 cells/well for biotinylation assays). Cultures were used at 12–15 DIV.

2.4. Plasmid constructs

GSK3 plasmids: prCMV-GSK3 and prCMV-K85RGSK3 (DN GSK3) were kindly provided by Dr. Felix Hernandez (Universidad Autónoma de Madrid). GLT-1 plasmid is derived from GLT-1 clone obtained from Dr. B. I. Kanner (2006) (The Hebrew University, Jerusalem) and the QuikChange Site-Directed Mutagenesis kit according to the manufacturer's instructions. pcDNA-GLAST plasmid (image clone 30102325) was purchased from GeneService. Previously, an HA tag was included at the N terminus of the transporters by PCR. HA-tagged forms of GLT-1 and GLAST have been previously used in the literature without any reported effect on the trafficking or activity of these transporters (Sheldon et al., 2006). For electrophysiological recordings, the cDNAs for GLT-1, GLAST, wtGSK3 and DN GSK3 were subcloned into the vector pSP64T, which contains the 5- and 3-UTRs of the *Xenopus laevis* globin gene (provided by Dr. Carmen Montiel, Universidad de Autónoma, Madrid). All constructs and mutants were confirmed by sequencing.

2.5. Expression in xenopus oocytes

The cDNAs cloned into pSP64T were linearized with *Xba*I (GSK3) or *Sal*I (GLAST and GLT-1), and the cRNAs were transcribed with SP6 polymerase and capped with 5, 7-methylguanosine using the mMESSAGE mMACHINE SP6 RNA kit (Ambion Inc.). *Xenopus laevis* were obtained from Xenopus Express (France), and oocytes were harvested from animals anesthetized in 0.1% (w/v) Tricaine methanesulfonate solution in tap water. All these procedures were performed in accordance with the Spanish and European guidelines for the prevention of cruelty to animals. The follicular membrane was removed by incubation in buffer (90 mM NaCl, 1 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.4) containing 300 units/ml collagenase (Type 1; Sigma) for 1 h. cRNA encoding either GLT-1 or GLT-1 + GSK3 GLT-1 + GSK3DN or GLAST or GLAST + GSK3 GLAST + GSK3DN (50 ng) were injected into defolliculated stage V and VI oocytes. The oocytes were maintained in Barth's medium