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Glycine transporters GlyT1 and GlyT2 are differentially modulated by glycogen synthase kinase 3β

Esperanza Jiménez [a, b, c], Enrique Núñez [a, b, c], Ignacio Ibáñez [a, b, c], Francisco Zafra [a, b, c], Carmen Aragón [a, b, c, 1], Cecilio Giménez [a, b, c, *, 1]

[a] Centro de Biología Molecular Severo Ochoa, Universidad Autónoma de Madrid, Consejo Superior de Investigaciones Científicas, 28049 Madrid, Spain
[b] Centro de Investigación Biomédica en Red de Enfermedades Raras, ISCIII, Madrid, Spain
[c] IdiPAZ-Hospital Universitario La Paz, Madrid, Spain

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ABSTRACT

Inhibitory glycinergic neurotransmission is terminated by the specific glycine transporters GlyT1 and GlyT2 which actively reuptake glycine from the synaptic cleft. GlyT1 is associated with both glycinergic and glutamatergic pathways, and is the main regulator of the glycine levels in the synapses. GlyT2 is the main supplier of glycine for vesicle refilling, a process that is vital to preserve the quantal glycine content in synaptic vesicles. Therefore, to control glycinergic neurotransmission efficiently, GlyT1 and GlyT2 activity must be regulated by diverse neuronal and glial signaling pathways. In this work, we have investigated the possible functional modulation of GlyT1 and GlyT2 by glycogen synthase kinase 3 (GSK3β). This kinase is involved in mood stabilization, neurodegeneration and plasticity at excitatory and inhibitory synapses. The co-expression of GSK3β with GlyT1 or GlyT2 in COS-7 cells and Xenopus laevis oocytes, leads to inhibition and stimulation of GlyT1 and GlyT2 activities, respectively, with a decrease of GlyT1, and an increase in GlyT2 levels at the plasma membrane. The specificity of these changes is supported by the antagonism exerted by a catalytically inactive form of the kinase and through inhibitors of GSK3β such as lithium chloride and TDZD-8. GSK3β also increases the incorporation of 32Pi into GlyT1 and decreases that of GlyT2. The pharmacological inhibition of the endogenous GSK3β in neuron cultures of brainstem and spinal cord leads to an opposite modulation of GlyT1 and GlyT2.Our results suggest that GSK3β is important for stabilizing and/or controlling the expression of functional GlyTs on the neural cell surface.

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1. Introduction

Inhibitory glycinergic neurotransmission is terminated by the specific glycine transporters GlyTs (GlyT1 and GlyT2) which actively reuptake glycine from the synaptic cleft. GlyTs belong to the neurotransmitter: sodium symporter family (*SLC6* gene family), which includes transporters for most of the neurotransmitters, serotonin, dopamine, norepinephrine and GABA, in the central nervous system (CNS) (Aragón and López-Corcuera, 2003). GlyT1 is associated with both glycinergic and glutamatergic pathways and is the main regulator of glycine levels in the synapses. The neuronal transporter GlyT2 recycles the neurotransmitter to the presynaptic terminal, a process that is absolutely necessary to preserve the quantal glycine content inside the synaptic vesicles (Apostolides and Trussell, 2013; Gomeza et al. 2003b; Harvey and Yee, 2013; Rousseau et al., 2008). Mouse gene disruption studies have revealed that constitutive deletion of either GlyT1 or GlyT2 is lethal (primarily as a result of excessive or deficient glycinergic inhibition, respectively), and suggest that alterations in GlyTs may underlie several human disorders (Aragón and López-Corcuera, 2005; Gomeza et al., 2003a, 2003b). These studies revealed the

Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; CNS, central nervous system; COS-7, CV-1 (simian) in origin and carrying the SV40 genetic material cells; DTT, dithiothreitol; EGTA, ethyleneglycoltetraacetic acid; EDTA, ethylenediaminetetraacetic acid; GABA, γ-aminobutyric acid; GlyT1, glycine transporter 1; GlyT2, glycine transporter 2; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NMDA, N-methyl-D-aspartic acid; PBS, phosphate buffer saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SLC6, solute carrier 6: TDZD-8, 4-benzyl-2-methyl-1, 2, 4-thiadiazolidine-3,5-dione.

* Corresponding author. Centro de Biología Molecular Severo Ochoa, Universidad Autónoma de Madrid, C / Nicolás Cabrera, 1, 28049 Madrid, Spain. Tel.: +34 91 196 4629.
E-mail address: cgimenez@cbm.csic.es (C. Giménez).
[1] These authors share last authorship.

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role of GlyTs as homeostatic regulators of glycine levels in glycinergic and glutamatergic pathways that controls the balance of neuronal excitation and inhibition within several neural circuits. GlyT1 and GlyT2 have been related to disorders of central and peripheral nervous system, such as schizophrenia, depression, epilepsy, obsessive-compulsive disorders, anxiety disorders, pain, alcohol dependence, breathing disorders and hereditary hyperekplexia (Harvey and Yee, 2013). Indeed, mutations in the gene encoding GlyT2 are the second main cause of hyperekplexia in humans (Carta et al., 2012; Eulenburg et al., 2006; Gimenez et al., 2012; Rees et al., 2006) and produce congenital muscular dystonia type 2 (CMD2) in Belgian Blue cattle (Gill et al., 2012). Moreover GlyT1 inhibitors may improve cognitive deficits of schizophrenia by increasing glycine levels around the NMDA receptors. GlyT1 inhibitors are being developed by the pharmaceutical industry, mostly intended for treatment of cortical NMDA receptor hypofunction in schizophrenia (Javitt, 2008; Pinard et al., 2010).

An analysis of knock-out animals proved that the modulation of glycine transporter expression and/or transport activity influenced glycine-mediated neurotransmission and opened a way to find therapeutic applications (Gomeza et al., 2003a, 2003b). The levels of active glycine transporters in the plasma membrane are controlled by several factors in a regulated manner (de Juan-Sanz et al., 2011; de Juan-Sanz et al., 2013a; de Juan-Sanz et al., 2013b; Fornes et al., 2008; Geerlings et al., 2001; Nuñez et al., 2008). In the CNS these regulatory pathways must be triggered by physiological stimuli or the activity of appropriate receptors (Jiménez et al., 2011).

Glycogen synthase kinase 3 (GSK3) is currently considered to be a multifunctional serine/threonine kinase involved in a wide spectrum of cellular processes such as glycogen metabolism, cell proliferation, neuronal function, oncogenesis or embryonic development (for recent reviews see: Rayasam et al., 2009; Wildburger and Laezza, 2012). Although the protein is expressed in nearly all tissues, its highest levels and activity are found in the CNS (Leroy and Brion, 1999; Woodgett, 1990). Two distinct, but closely related forms of GSK3, GSK3α and GSK3β, have been identified. GSK3 is constitutively active in resting cells and its activity can be inhibited by phosphorylation at serine residues (Ser21 for GSK3α and Ser9 for GSK3β) on their N-terminal domain. By controlling the phosphorylation of these residues, neurons regulate GSK3 activity (for review see Doble and Woodgett, 2003). In animal models, the overexpression of GSK-3 induces increased vulnerability to mood-related behavioral disturbances and impaired socialization behavior (Mines et al., 2010; Polter et al., 2010). Furthermore, in clinical studies changes in the expression and activity of GSK-3 are found in schizophrenia (Emamian, 2012; Jope, 2003; Kozlovsky et al., 2001, 2002; Lovestone et al., 2007), mood disorders (Eldar-Finkelman, 2002; Jope, 2011), addictive behaviors (Miller et al., 2009, 2010) and Alzheimer's disease (Balaramanetal., 2006; Hooper et al., 2008; Kremer et al., 2011). Recently, the role of GSK3β has emerged in the pathogenesis of pain (Maixner et al., 2014).

Despite the pleiotropic effects of GSK3, or probably because of them, many of their molecular targets in the CNS have not yet been identified. Lately, GSK-3 has been proposed as a key element in plasticity at excitatory and inhibitory synapses in the CNS (Bradley et al., 2012). The molecular mechanisms underlying, at least partially, the role of GSK-3 in synaptic plasticity is through the regulation of NMDA and AMPA receptors endocytosis (Bradley et al., 2012; Chen et al., 2007; Wei et al., 2010).

Given the essential role of plasma membrane trafficking to control GlyTs activity in excitatory and inhibitory synapsis and the fact that GSK3 and glycine transporters share implication in some CNS disorders such as, neuropathic pain, schizophrenia and alcohol dependence, in this work we have investigated whether GSK3 is involved in the modulation of these transporters. Our results provide evidence of a differential regulation of GlyT1 and GlyT2 by GSK3β in heterologous and neuronal cells.

2. Materials and methods

2.1. Materials

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.

[^{3}H]Glycine (PERKIN ELMER) protein standards for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Precision Plus Standards) (Bio-Rad), and the Enhanced Chemiluminescence (ECL) Western blotting detection reagents were obtained from Amersham. GlyT2 inhibitor ALX-1393 O-[(2-Benzyloxyphenyl-3-flurophenyl) methyl]-L-serine was purchased from Sigma. NFPS (ALX-5407) hydrochloride, N-[3-(4′-Fluorophenyl)-3-(4′-phenylphenoxy) propyl]sarcosine hydrochloride was obtained from Axon Medchem. True-Fect-LipoTM was from United Biosystems (Rockville, MD), EZ-Link Sulfo–NHS–SSBiotin was from Pierce. pcDNA3 plasmid was purchased from Invitrogen, the Expand High Fidelity PCR system (Taq polymerase) and all restriction enzymes were obtained from Roche Applied Science. The pGEM-T easy cloning vector was purchased from Promega (Madison, WI) and the oligonucleotides used were synthesized by Sigma. The QuikChange Site-Directed Mutagenesis kit was from Stratagene (La Jolla, CA), nitrocellulose sheets were from Bio-Rad and fetal calf serum was supplied by Invitrogen.

Rat antibody against GlyT2 and rabbit antibody against GlyT1 have been previously characterized (Zafra et al., 1995). Antibody against GSK3β were from BD Transduction Laboratories. Anti-rabbit and anti-rat coupled to AlexaFluor® 555 fluorophore for GlyT1 and GlyT2 and anti-mouse antibody coupled to AlexaFluor® 488 for GSK3β secondary antibodies were used.

All other chemicals were obtained from Sigma.

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% fetal bovine serum at 37 °C in an atmosphere of 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. Plasmid constructs

GSK3 plasmids: prCMVGSK3β and prCMV K85R GSK3β (DN GSK3β) were kindly provided by Dr Felix Hernandez (Centro de Biología Molecular Severo Ochoa, Universidad Autónoma de Madrid, Spain). GlyT1 and GlyT2 cDNAs were subcloned into pcDNA3, as described (Smith et al., 1992; Liu et al., 1993).

For electrophysiological recordings, the cDNAs for GlyT1, GlyT2, wt GSK3β, and DN GSK3β 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 Autónoma de Madrid). All constructs and mutants were confirmed by sequencing.

2.4. Expression in Xenopus oocytes

The cDNAs cloned into pSP64T were linearized with XbaI (GSK3) or SalI (GlyT2 and GlyT1) and the cRNAs were transcribed with SP6 polymerase and capped with 5,7-methylguanosine using the mMESSAGE mMACHINE SP6 RNA kit (Ambion Inc.). *X. laevis* frogs were obtained from Xenopus Express (France) and oocytes were harvested from *X. laevis* 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 a medium (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. cRNAs encoding either GlyT1 or GlyT1:GSK3β, GlyT1:GSK3β DN or GlyT2 or GlyT2:GSK3β, GlyT2:GSK3β DN (50 ng) were injected into defolliculated stage V and VI *X. laevis* oocytes. The oocytes were maintained in Barth's medium (88 mM NaCl, 1 mM KCl, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 2.4 mM $NaHCO_3$, 10 mM HEPES (pH7.4) and transport or electrophysiological experiments were carried out 5 days later.

2.5. Two-microelectrode voltage clamp recordings of Xenopus oocytes

Electrophysiological recordings were obtained after incubating the injected oocytes at 18 °C in standard oocyte solution (100 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, pH adjusted to 7.5 with HCl). A two-electrode voltage clamp was used to measure and control the membrane potential and to monitor the capacitative currents using Axoclamp 900A (Axon Instruments). The two electrode signals were digitized by a Digidata 1440A (Axon Instruments) and both instruments were controlled by the pCLAMP software (Axon Instruments). The results were analyzed by Clampfit 10.2 software (Axon Instruments). The

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