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BDNF modulates glycine uptake in hippocampal synaptosomes by decreasing membrane insertion of glycine transporter 2

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ABSTRACT

Glycine transporter 2 (GlyT2) is localized in the nerve terminals of glycinergic neurons, promoting glycine uptake and ensuring the refilling of glycinergic vesicles. Brain-derived neurotrophic factor (BDNF) activates its high affinity TrkB receptors, which occur in two isoforms, full length (TrkB-FL) and truncated (TrkB-T1/T2). After BDNF binding to TrkB receptor, several intracellular cascades are triggered, specifically PLC, Akt and MAPK signalling pathways.

We herein show that BDNF decreases [^{3}H]glycine uptake mediated by GlyT2 in isolated nerve endings (synaptosomes) obtained from rat hippocampus, by reducing the maximum velocity (V_{max}) of transport while not influencing the transporter affinity constant (K_m) for glycine. Western Blot analysis detected both TrkB receptor isoforms in the synaptosomes but the BDNF effect seems to be mediated by TrkB-FL since: 1) the tyrosine kinase inhibitor, k252a, prevented the effect of BDNF, and 2) the effect of BDNF was lost in the presence of specific inhibitors of TrkB signalling pathways, namely U73122, LY294002 and U0126 (inhibitors of PLC, Akt and MAPK pathways, respectively). Monensin, a transporter recycling inhibitor, prevented the BDNF action upon glycine uptake, suggesting that BDNF reduces GlyT2 insertion in the plasma membrane.

It is concluded that BDNF effect upon glycine uptake into glycinergic nerve terminals requires the activation of the TrkB-FL receptor and its canonical signalling pathways and occurs by inhibiting GlyT2 membrane incorporation.

1. Introduction

Glycine and GABA are considered the main inhibitory neurotransmitters in the mammalian central nervous system (CNS). Glycine activates the inhibitory glycine receptors (GlyR) and it also serves as an essential co-agonist of excitatory *N*-methyl-*d*-aspartate (NMDA) receptors (Johnson and Ascher, 1987). The levels of extracellular glycine are regulated through the activity of two specific Na^+/Cl^--dependent plasma membrane glycine transporters (GlyT). Two types of GlyT have been identified, namely GlyT1 (Guastella et al., 1992) and GlyT2 (Liu et al., 1992). GlyT1 and GlyT2 have different cellular distributions, which leads to different functions in synapses (Eulenburg et al., 2005). GlyT1 is expressed in astrocytes and glutamatergic nerve endings (Eulenburg et al., 2005). Glial GlyT1 eliminates glycine from the synaptic cleft and therefore regulates glycine concentrations at inhibitory synapses, whereas neuronal GlyT1 regulates the concentration of glycine at excitatory synapses containing NMDA receptors (Eulenburg et al., 2005; Betz et al., 2006). GlyT2, the focus of this study, is largely localized to

Abbreviations: aCSF, artificial cerebrospinal fluid; Akt, protein kinase B; BDNF, brain-derived neurotrophic factor; BSA, bovine serum albumin; CNS, central nervous system; Erk, extracellular signal-regulated kinases; FBS, fetal bovine serum; GABA, gamma-aminobutyric acid; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GAT, GABA transporters; GFAP, glial fibrillary acidic protein; GlyR, glycine receptors; GlyT1, glycine transporter 1; GlyT2, glycine transporter 2; HRP, horseradish peroxidase; KHR solution, Krebs-Henseleit-Ringer solution; K_m, affinity constant of Michaelis-Menton model; MAP2, microtubule-associated protein 2; MAPK, mitogen-associated protein kinase; NMDA, *N*-methyl-D-aspartate; PBS, phosphate buffered saline; PFA, paraformaldehyde; PI3K, phosphatidylinositol-3 kinase; PLCγ, phospholipase C-γ; PKC, protein kinase C; RT, room temperature; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SEM, standard error of the mean; SV2, synaptic vesicle 2; TBS-T, tris-buffered saline with tween-20; TrkB, tropomyosin-related kinase B receptors; TrkB-FL, TrkB full length receptors; TrkB-T, TrkB truncated receptors; V_{max}, maximum velocity of transport.

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glycinergic neurons (Zafra et al., 1995; Eulenburg et al., 2005) and has a crucial role in glycine uptake into the cytosolic space, thus ensuring the refilling of presynaptic vesicles (Gomeza et al., 2003b). Indeed, GlyT2 is regarded as a marker for glycine-containing nerve endings (Poyatos et al., 1997). Recently, it was demonstrated that functional GlyT2 was also expressed in cortical astrocytes, although with a much lower affinity for glycine than glial GlyT1, most probably contributing, together with astrocytic GlyT1, to shape glycinergic transmission (Aroeira et al., 2014). In fact, low affinity transporters are described to play a relevant role in glycinergic transmission, since synaptic levels of glycine may transiently increase to the millimolar range (Dohi et al., 2009).

Brain-derived neurotrophic factor (BDNF) belongs to the neurotrophin family and has important functions in neuronal differentiation, maturation and survival, as well as synaptic transmission and plasticity modulation (Binder and Scharfman, 2004). BDNF can activate two categories of receptors, the tropomyosin-related kinase (Trk) receptor and the p75 neurotrophin (p75NTR) receptor (Patapoutian and Reichardt, 2001; Roux and Barker, 2002).

The predominant TrkB receptor, the full length receptor (TrkB-FL) triggers, through the autophosphorylation of tyrosine residues in the intracellular kinase domain, the well-characterized mitogen-associated protein kinase (MAPK), phosphatidylinositol-3 kinase (PI3K)/protein kinase B (Akt), and phospholipase C-γ (PLC-γ)/protein kinase C (PKC) signalling pathways. Truncated TrkB receptors, namely TrkB-T1, TrkB-T2 and TrkB-T-Shc, originated from alternative splicing, lack the intracellular kinase domain (Stoilov et al., 2002; Binder and Scharfman, 2004; Blum and Konnerth, 2005). At nerve endings of the hippocampus, BDNF has been shown to modulate glutamate and GABA release (Canas et al., 2004; Jerónimo-Santos et al., 2015) and to regulate GABA transporter 1 (GAT-1) activity (Vaz et al., 2008), through TrkB-FL receptors. Recently, the potential role of truncated TrkB receptor in mediating the BDNF effect has been suggested (Ohira et al., 2005; Fenner, 2012). It was described that in astrocytes, TrkB-T receptor stimulates the PLC pathway, leading to a BDNF-dependent Ca^{2+} release (Rose et al., 2003) and activation of the PKC intracellular cascade (Cheng et al., 2007). Furthermore, it was suggested that TrkB-T1 receptors are important regulators of TrkB-FL signalling *in vivo* (Carim-Todd et al., 2009). We also reported that glycine transport into astrocytes is inhibited by BDNF, which by activating TrkB-T1 receptors, promotes GlyT1 and GlyT2 internalization through a Rho-GTPase activity dependent mechanism (Aroeira et al., 2015). In contrast, BDNF was described to facilitate GABA transport into astrocytes, this action being also mediated by TrkB-T receptors (Vaz et al., 2011).

GlyT activity's dysregulation was shown to be involved in some human disorders, including neuromotor deficiencies (startle disease, myoclonus), pain and epilepsy (Gomeza et al., 2003a, 2003b; Aragon and Lopez-Corcuera, 2005). Hence, a better understanding of the mechanisms that modulate GlyT2 function can uncover potential therapeutic targets for the treatment of these conditions.

In the work herein reported we evaluated the role of BDNF upon GlyT2 activity in nerve terminals isolated from the rat hippocampus.

2. Materials and methods

2.1. Animals

Sprague-Dawley rats (4 weeks old) were acquired from Harlan (Barcelona, Spain). All procedures were performed according to the Portuguese Laws and with the European guidelines (2010/63/EU), being approved by the Ethics Commission of the Faculty of Medicine, University of Lisbon. All the experimental work was carried out with the lowest amount of animals and in order to minimize animal pain and suffer. Animals were deeply anesthetized with 2-chloro-2-(difluoromethoxy)-1,1,1-trifluoro-ethane before decapitation for brain removal.

2.2. Reagents and drugs

Unless stated otherwise all reagents were purchased from Sigma (St. Louis, MO, USA).

[^{3}H]glycine (specific activity 44.7 Ci/mmol) was acquired from PerkinElmer (Waltham, MA, USA).

BDNF was gently supplied by Regeneron Pharmaceuticals (Tarrytown, NY). Org 24598, a specific GlyT1 inhibitor (Brown et al., 2001) and ALX 1393 (Luccini and Raiteri, 2007), a specific GlyT2 inhibitor, were purchased from Sigma. K252a was obtained from Calbiochem (Billerica, MA, USA). LY 294002 and U0126 were achieved from Ascent (Weston-Super-Mare, UK), while U73122 and U0124 were purchased from Tocris (Bristol, UK).

2.3. Antibodies

For western blot, the primary antibodies used were mouse IgG1 antibody to TrkB (BD Biosciences, Franklin Lakes, NJ, USA, 1:1000) and mouse monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Life Technologies Corporation, Carlsbad, CA, USA, 4 mg/ml, 1:10000). The HRP-coupled secondary antibody (Santa Cruz, Santa Cruz, CA, USA, 1:10000) used was goat anti-mouse.

For immunocytochemistry, the primary antibodies used were rabbit antibody anti-GlyT2 (Alpha Diagnostic Intl. Inc., San Antonio, TX, USA, 1:20), rabbit antibody anti-glial fibrillary acidic protein (GFAP), a known marker for differentiated astrocytes (Sigma, 1:500), mouse monoclonal AP20 anti-Microtubule Associated Protein 2 (MAP2) (Chemicon, Temecula, CA, USA, 1 mg/ml, 1:250), a neuronal marker, and mouse monoclonal 246E8 anti-Synaptic Vesicle 2 (SV2) (Synaptic Systems, Göttingen, Germany, 1:250), integral membrane glycoproteins highly abundant in synaptic vesicles. The fluorescent-labelled secondary antibodies used were goat anti-rabbit-Alexa 568 and goat anti-mouse-Alexa 488 (Invitrogen, Grand Island, NY, USA, 1:400).

2.4. Synaptosomes preparation

After decapitation, the brain was quickly removed into oxygenated (95% O_2 and 5% CO_2) and ice-cold artificial cerebrospinal fluid (aCSF) (in mM: NaCl 124, KCl 3, NaH_2PO_4 1.2, $NaHCO_3$ 25, $CaCl_2$ 2, $MgSO_4$ 1 and glucose 10, pH 7.4). The hippocampi/brainstem were dissected out and added to 15 ml of a chilled sucrose solution (sucrose 0.32 M in EDTA 1 mM, HEPES 10 mM, 1 mg/ml BSA, pH 7.40). After homogenization at 4 °C and centrifugation (1000 g, 10 min, 4 °C) (Heraeus sepatech – Biofuge 28RS centrifuge), the supernatant was collected and centrifuged for a second time (14000 g, 12 min, 4 °C). The pellet, which corresponds to the membrane fraction, was collected and ressuspended in 3 ml Percoll solution (Percoll 45% (v/v) in KHR solution: NaCl 140 mM, EDTA 1 mM, HEPES 10 mM, KCl 5 mM and glucose 5 mM, pH 7.40). This suspension was centrifuged (14 000 g, 2 min, 4 °C) and the top layer containing the synaptosomal fraction, was removed, washed with KHR and centrifuged (14 000 g, 2 min, 4 °C) for two times. The pellet was ressuspended in chilled Krebs-HEPES solution (glucose 10 mM, NaCl 125 mM, KCl 3 mM, $MgSO_4$ 1.2 mM, NaH_2PO_4 1 mM, $CaCl_2$ 1.5 mM and HEPES 10 mM, pH 7.4) and kept at 4 °C. Soluble protein was quantified according with the Bradford method (Bradford, 1976).