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FUNCTIONAL EXPRESSION OF TWO SYSTEM A GLUTAMINE TRANSPORTER ISOFORMS IN RAT AUDITORY BRAINSTEM NEURONS

A. BLOT,[a] D. BILLUPS,[a] M. BJØRKMO,[b,c] A. Z. QUAZI,[b,c] N. M. UWECHUE,[a] F. A. CHAUDHRY[b,c] AND B. BILLUPS[a]*

[a]*Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1PD, UK*

[b]*The Biotechnology Centre of Oslo, University of Oslo, Blindern, N-0317 Oslo, Norway*

[c]*The Centre for Molecular Biology and Neuroscience, University of Oslo, Blindern, N-0317 Oslo, Norway*

Abstract—Glutamine plays multiple roles in the CNS, including metabolic functions and production of the neurotransmitters glutamate and GABA. It has been proposed to be taken up into neurons via a variety of membrane transport systems, including system A, which is a sodium-dependent electrogenic amino acid transporter system. In this study, we investigate glutamine transport by application of amino acids to individual principal neurons of the medial nucleus of the trapezoid body (MNTB) in acutely isolated rat brain slices. A glutamine transport current was studied in patch-clamped neurons, which had the electrical and pharmacological properties of system A: it was sodium-dependent, had a non-reversing current-voltage relationship, was activated by proline, occluded by N-(methylamino)isobutyric acid (MeAIB), and was unaffected by 2-aminobicyclo-[2.2.1]-heptane-2-carboxylic acid (BCH). Additionally, we examined the expression of different system A transporter isoforms using immunocytochemical staining with antibodies raised against system A transporter 1 and 2 (SAT1 and SAT2). Our results indicate that both isoforms are expressed in MNTB principal neurons, and demonstrate that functional system A transporters are present in the plasma membrane of neurons. Since system A transport is highly regulated by a number of cellular signaling mechanisms and glutamine then goes on to activate other pathways, the study of these transporters *in situ* gives an indication of the mechanisms of neuronal glutamine supply as well as points of regulation of neurotransmitter production, cellular signaling and metabolism in the native neuronal environment.

Key words: Slc38a1, Slc38a2, SAT1, SAT2, SNAT1, SNAT2.

*Corresponding author. Tel: +44-1223-334032; fax: +44-1223-334100.
E-mail address: bjb41@cam.ac.uk (B. Billups).

Abbreviations: APV, DL-2-amino-5-phosphonopentanoic acid; BCH, 2-aminobicyclo-[2.2.1]-heptane-2-carboxylic acid; DL-TBOA, DL-threo-b-benzyloxyaspartate; EAAT, excitatory amino acid transporter; GST, glutathione-S-transferase; I/V, current–voltage; I_{gln}, glutamine-induced current; I_{pro}, proline-induced current; MeAIB, N-(methylamino)isobutyric acid; MK801, dizocilpine maleate; MNTB, medial nucleus of the trapezoid body; NBQX, 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide; SAT1, system A transporter 1; SAT2, system A transporter 2; TCA, tricarboxylic acid; TTX, tetrodotoxin; VGLUT, vesicular glutamate transporter.

Glutamine is the most abundant amino acid in the interstitial fluid of the CNS, with a concentration in the range of 0.2–0.5 mM (Jacobson et al., 1985; Reichel et al., 1995; Kanamori and Ross, 2004), which is over an order of magnitude higher than any other amino acid. It is not known to have any direct neurotransmitter action, but does play several important roles within the CNS including production of proteins, pyrimidine nucleotides, excitatory and inhibitory neurotransmitters and tricarboxylic acid (TCA) cycle intermediates. The intracellular glutamine concentration is thought to be in the millimolar range, and transport into neurons occurs against a substantial concentration gradient, which requires energy-dependent transport proteins (Erecinska and Silver, 1990).

The main transport systems for glutamine are systems A, ASC, B^0, $B^{0,+}$, $b^{0,+}$, L, N, and y^+L (reviewed by Bode, 2001; Broer, 2008). Systems ASC, $b^{0,+}$, L and y^+L are obligate exchangers, swapping internal and external amino acids across the plasma membrane. In contrast, systems A, B^0, and $B^{0,+}$ use the energy derived by the sodium gradient to power the uptake of amino acids in an electrogenic manner, without counter-transporting another amino acid. System N co-transports sodium and counter-transports a proton, resulting in a transport process that is close to energetic equilibrium and can mediate the uptake or release of amino acids under physiological conditions (Chaudhry et al., 1999; Broer et al., 2002). Glutamine transporter expression in the CNS has been observed for system A isoforms SAT1 (also called SA2, SNAT1, NAT2, GlnT and ATA1: Varoqui et al., 2000; Wang et al., 2000; Chaudhry et al., 2002b; Melone et al., 2004) and SAT2 (also called SA1, SNAT2 and ATA2: Reimer et al., 2000; Sugawara et al., 2000a; Yao et al., 2000; Gonzalez-Gonzalez et al., 2005; Melone et al., 2006; Jenstad et al., 2009); system ASC (ASCT2: Broer et al., 1999; Dolinska et al., 2004; Yamamoto et al., 2004); system B^0 (B0AT2: Inoue et al., 1996; Takanaga et al., 2005; Broer et al., 2006); system $B^{0,+}$ ($ATB^{0,+}$: Sloan and Mager, 1999); system $b^{0,+}$ (rBAT/$b^{0,+}$AT: Bertran et al., 1992; Tate et al., 1992; Wells and Hediger, 1992); system L (LAT1 and LAT2: Kanai et al., 1998; Rossier et al., 1999; Segawa et al., 1999); system N (SN1/SNAT3 and SN2/SNAT5: Chaudhry et al., 1999, 2001; Nakanishi et al., 2001; Boulland et al., 2002, 2003; Cubelos et al., 2005) and system y^+L (y^+LAT2: Broer et al., 2000).

As most studies use radiolabelled substrates to study the properties of transport systems in bulk tissue or cultured cells, the cellular localization and functional properties of these different transporters *in vivo* is uncertain. To

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investigate glutamine transport in individual, identified neurons in their native physiological environment, we recorded amino acid-induced transporter currents using whole-cell patch-clamping in acutely isolated rat brain slices. *In situ* recordings were made from principal neurons in the medial nucleus of the trapezoid body (MNTB), which are neurons in the auditory brainstem that receive mainly excitatory glutamatergic input, and release glycine, GABA and glutamate at synapses in the adjacent medial and lateral superior olives (MSO and LSO; Spangler et al., 1985; Adams and Mugnaini, 1990; Bledsoe et al., 1990; Chaudhry et al., 1998; Gillespie et al., 2005). These cells have a spherical cell body with only a small dendritic tree (Smith et al., 1998; Leao et al., 2008), which allows for precise recording of somatic currents and eliminates artifacts due to dendritic filtering (Williams and Mitchell, 2008). The astrocytes surrounding the principal neurons in the MNTB have been shown to strongly express the system N transporters SN1 and SN2 (Boulland et al., 2002; Cubelos et al., 2005), which are thought to be responsible for the export of glutamine from the glial compartment (Chaudhry et al., 1999). System N and system A transporters in adjacent cells have been proposed to form a system N–system A shuttle (Chaudhry et al., 2002a; Gammelsaeter et al., 2009; Jenstad et al., 2009), which would transfer glutamine from glia to neurons. In support of this hypothesis, our electrophysiological and immunocytological data show that MNTB principal neurons express functional system A glutamine transporters on their soma. This provides a valuable insight into the possible mechanisms that these neurons employ for amino acid and neurotransmitter metabolism.

EXPERIMENTAL PROCEDURES

Slice preparation

Brain slices were obtained from 10 to 15 day old Wistar rats, killed by decapitation in accordance with the UK Animals (Scientific Procedures) Act 1986. All animal experiments were approved by the relevant local authorities (University of Cambridge, UK and University of Oslo, Norway), and every effort was taken to reduce the number of animals used and to minimize any suffering. Brains were quickly removed into a solution at approximately 0 °C containing (in mM) 250 sucrose, 2.5 KCl, 10 glucose, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 4 $MgCl_2$, 0.1 $CaCl_2$; gassed with 95% O_2 +5% CO_2 (pH 7.4). Transverse brainstem slices, approximately 150 μm thick, were cut using an Integraslice 7550PSDS (Campden Instruments, Loughborough, UK), and slices were placed in an incubation chamber maintained at 37 °C for half an hour, before being allowed to cool to room temperature and used within the next 6 h. The incubation chamber contained artificial cerebrospinal fluid (aCSF), which comprised (in mM) 125 NaCl, 2.5 KCl, 10 glucose, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 1 $MgCl_2$, 2 $CaCl_2$; gassed with 95% O_2 +5% CO_2 (pH 7.4).

Electrophysiological recording

MNTB neurons were visualized with infrared differential interference contrast (IR–DIC) optics on a Nikon E600FN microscope (Nikon Corporation, Tokyo, Japan) with a 60×, numerical aperture 1.0, water immersion, fluor lens. Slices were perfused at a rate of approximately 1 ml/min with aCSF (as above) at a temperature of 31–35 °C. In all experiments, except for Fig. 4A–C, a cocktail of channel inhibitors was added to the recording solution, containing (in μM) 40 dl-2-amino-5-phosphonopentanoic acid (APV), 10 dizocilpine maleate (MK801), 10 bicuculline, 1 strychnine, 1 TTX (tetrodotoxin) and 20 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo-[f]quinoxaline −7-sulfonamide (NBQX). In the experiments recorded in 0 mM sodium solution (Fig. 3), NaCl and $NaHCO_3$ were replaced by choline chloride and choline bicarbonate, respectively, and NaH_2PO_4 was replaced by KH_2PO_4 (with a corresponding reduction in KCl concentration and increase in choline chloride concentration to balance the other ions). Whole-cell patch-clamp recordings were made from MNTB cells using thick-walled glass pipettes (GC150F-7.5; Harvard Apparatus, Edenbridge, Kent, UK) with a HEKA EPC-10 double amplifier and Patchmaster software (HEKA Elektronik Dr. Schulze GmbH; Lambrecht/Pfalz, Germany). The intracellular solution contained (in mM) 110 Cs-methanesulfonate, 40 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES), 10 tetraethylammonium chloride (TEA), 5 Na_2-phosphocreatine, 20 sucrose, 0.2 ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), 2 MgATP, 0.5 NaGTP, and 8 μM $CaCl_2$ (pH 7.2 with ~10 mM CsOH). A measured liquid junction potential of 3 mV was not corrected for. Recording pipette open-tip resistances were 5–8 MΩ and whole-cell access resistances were <30 MΩ. Lucifer Yellow (<0.05%) was added to the internal solution to allow confirmation of the post synaptic recording site by fluorescent imaging following the completion of the experiment. MNTB cells were voltage-clamped at −70 mV (unless stated) and currents were filtered at 10 and 2.9 kHz, and digitized at 25 kHz. Glutamine (or other amino acids) were dissolved in the relevant external solution and applied by pressure ejection (5–10 p.s.i.) from a patch pipette (as above, open tip resistance 4–6 MΩ) using a Picospritzer II (General Valve, Fairfield, NJ, USA). Puff applications were repeated at 30 s intervals. When applying two different glutamine concentrations, or glutamine in different external solutions, two puffer pipettes were used. The pipettes were a pair pulled from one piece of glass and therefore had the same tip diameter, were attached to the same pressure source and were placed equidistant from the cell.

Immunostaining

Male Wistar rats, 200–300 g, were anesthetized with pentobarbital and subjected to transcardial perfusion fixation with 4% paraformaldehyde in 0.1 M sodium phosphate buffer pH 7.4. The brains were dissected out and kept in fixative over night. 40–50 μm thick coronal sections were cut by a vibratory microtome. Immunoperoxidase staining was done as described previously (Chaudhry et al., 1998; Boulland et al., 2004). Briefly, free-floating sections were treated with 1 M ethanolamine (pH 7.4), followed by incubation with 1% H_2O_2 in phosphate-buffered saline (PBS). The sections were incubated in solution of 10% fetal bovine serum and 0.1% NaN_3 in buffer A (0.3 M NaCl, 0.1 M Tris-HCl pH 7.4, 0.05% Triton X-100) prior to incubation with the primary antibodies in the same solution. Following incubation in biotinylated secondary antibodies and the streptavidin-biotinylated horseradish peroxidase complex, the immunoreaction was demonstrated by addition of 3,3′-diaminobenzidine (0.5 mg/ml) in PBS activated by H_2O_2.

The affinity-purified antibodies against SAT1 and SAT2 have been characterized and were used as described previously (Buntup et al., 2008; Jenstad et al., 2009). SAT1 was used at a final concentration of 1 μg/ml, while SAT2 was used at a concentration of 10 μg/ml. The specificity of the immunoreaction was confirmed by pre-incubating the antibodies with the corresponding glutathione-S-transferase (GST) fusion protein (30–100 μg) used for immunization of the rabbits.

For fluorescent immunocytochemistry, 13–14 day old rats were used and stained as previously described (Billups, 2005). SAT1 and SAT2 antibodies (as above) were used at 0.5 and 0.25 μg/ml respectively, and secondary fluorescent antibodies (Alexa Fluor 488, 2 μg/ml; Invitrogen, Carlsbad, CA, USA) were visualized with a confocal microscope (Leica SP5; Leica Microsystems