Localization and Functional Relevance of System A Neutral Amino Acid Transporters in Cultured Hippocampal Neurons*

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Glutamine and alanine are important precursors for the synthesis of glutamate. Provided to neurons by neighboring astrocytes, these amino acids are internalized by classical system A amino acid carriers. In particular, System A transporter (SAT1) is a highly efficient glutamine transporter, whereas SAT2 exhibits broad specificity for neutral amino acids with a preference for alanine. We investigated the localization and the functional relevance of SAT1 and SAT2 in primary cultures of hippocampal neurons. Both carriers have been expressed since early developmental stages and are uniformly distributed throughout all neuronal processes. However, whereas SAT1 is present in axonal growth cones and can be detected at later developmental stages at the sites of synaptic contacts, SAT2 does not appear to be significantly expressed in these compartments. The non-metabolizable amino acid analogue α-(methylamino)-isobutyric acid, a competitive inhibitor of system A carriers, significantly reduced miniature excitatory postsynaptic current amplitude in neurons growing on top of astrocytes, being ineffective in pure neuronal cultures. α-(Methylamino)-isobutyric acid did not alter neuronal responsibility to glutamate, thus excluding a postsynaptic effect. These data indicate that system A carriers are expressed with a different subcellular distribution in hippocampal neurons and play a crucial role in controlling the astrocyte-mediated supply of glutamatergic neurons with neurotransmitter precursors.

Glial cells, which represent a large percentage of the cell population in the brain, are intimately juxtaposed to neurons throughout the nervous system. Once considered as a purely structurally supportive element for neurons, astrocytes are now recognized as active neuronal partners, which participate in the formation of the synapse and dynamically control neuronal activity and synaptic transmission (1–4).

One of the major roles played by astrocytes is to control glutamate concentration in the synaptic cleft. The astrocytic glutamate transporters glutamate/aspartate transporter and L-glutamate transporter are responsible for most of the clearance of synaptically released glutamate from the extracellular space (5, 6). Once accumulated into astrocytes, glutamate is converted to the non-excitatory amino acid glutamine by the glial enzyme glutamine synthetase. Glutamine is then returned to neurons and used as a source for the synthesis of glutamate (7–9) through its conversion by phosphate-activated glutaminase (reviewed in Refs. 1 and 2). A second major metabolic fate of glutamate once accumulated by astrocytes is a reaction with pyruvate to form alanine via the alanine aminotransferase pathway (10). As amino group donor, alanine together with α-ketoglutarate is efficiently converted to glutamate via a transamination reaction in neurons (11, 12). As both α-ketoglutarate and alanine are supplied to neurons by astrocytes (13, 14), it is conceivable that glutamatergic neurons are dependent upon neighboring astrocytes for the supply of these glutamate precursors in addition to glutamine.

Glutamine and alanine are thought to efflux from astrocytes via specific carriers belonging to the System N family and to be accumulated by neurons via specific carriers belonging to the System A family of neutral amino acid transporters (reviewed in Ref. 15). System A transporters are sodium-dependent and unidirectional, mediating Na⁺−coupled cellular uptake of small aliphatic amino acids such as alanine, serine, and glutamine. System A transporters are distinguished from the other amino acid transporter systems, L, ASC, and N, by its ability to transport N-methylated substrates such as the non-metabolizable amino acid analogue α-(methylamino)-isobutyric acid (MeAIB) and by its property of being regulated by a variety of environmental conditions (16, 17). Two members of the system A family have been recently cloned. SAT1, previously called GlnT, is a highly efficient glutamine transporter (18), and SAT2 exhibits a broader specificity for neutral amino acids (19–21). In the central nervous system, both SAT1 and SAT2 are enriched on neuronal cells (18, 20, 21), whereas the system N transporter (SN1) is restricted to astrocytes (22).

To evaluate the roles SAT1 and SAT2 play as molecular gateways to provide precursors for the synthesis of glutamate in neuronal cells, we have investigated their distribution and function in primary cultures of developing hippocampal neurons, a widely used model for the study of glutamatergic transmission.

EXPERIMENTAL PROCEDURES

Pure Neuronal Cultures—Primary neuronal cultures were prepared from the hippocampi of 18-day-old fetal rats as described previously (23).
24). Hippocampi were dissociated by treatment with 0.25% trypsin for 15 min at 37 °C followed by trituration with a fire-polished Pasteur pipette. Dissociated cells were plated on poly-L-lysine-treated (Sigma) glass coverslips in minimum Eagle's medium with 10% horse serum at densities ranging from 10,000 cells/cm² to 20,000 cells/cm². After few hours, coverslips were transferred to dishes containing a monolayer of cortical glial cells (25), so that they were suspended over the glial cells but not in direct contact with them (24). Cells were maintained in minimum Eagle's medium (Invitrogen Italia-Life Technologies, Milano, Italy) serum free supplemented with 1% N2 (Invitrogen), 2 mM glutamine, and 1 mg/ml bovine serum albumin (neuronal medium) (26).

Antibodies—Conjugated antibodies were from Jackson ImmunoResearch Laboratories, protein (Sigma). Anti-rabbit rhodamine-conjugated antibodies were purchased from Molecular Probes (Eugene, OR). The primary antibodies used were polyclonal antiserum against the carrier proteins in the central nervous system (Fig. 1) (18, 21). No immunoreactivity for either SAT1 or SAT2 was detectable in cell extracts from pure astrocytic cultures, the anti-SAT1 and anti-SAT2 antibodies recognized single bands with the molecular mass (about 55 kDa) previously defined for the two carrier proteins in the central nervous system (Fig. 1) (18, 21). No immunoreactivity for either SAT1 or SAT2 was detectable in cell extracts from pure astrocytic cultures (data not shown) (18, 21).

The development of hippocampal neurons in culture follows a predictable sequence of events (29). When maintained in a medium conditioned by the presence of a monolayer of cortical astrocytes, which although not in contact with neurons provide factors necessary for their survival and differentiation, cells initially extend several equivalent minor processes. In the next few hours, one of these processes, the axon, begins growing rapidly and selectively, and eventually after 7–10 days, the axon becomes able to form physiologically active axo-somatic and axo-dendritic synapses (24). Both SAT1 (Fig. 2, A and C) and SAT2 (Fig. 2, E and G) were expressed in hippocampal neurons at early stages of neuronal development (3 days in vitro). Both carriers appeared to be diffusely distributed in all neuronal compartments including axons (see Fig. 2, B and F, double stainings with antibodies against the synaptic vesicle protein synaptobrevin/VAMP2). However, whereas SAT1 im-

**RESULTS**

Expression and Localization of SAT1 and SAT2 in Hippocampal Cultures—We have used previously characterized polyclonal antibodies directed against the NH₂-terminus of SAT1 (18) or against the NH₂-terminus of SAT2 (21) to investigate the distribution of these carriers in primary cultures of hippocampal neurons. When tested on homogenates of rat hippocampi as well as on cell extracts of mature hippocampal cultures, the anti-SAT1 and anti-SAT2 antibodies recognized single bands with the molecular mass (about 55 kDa) previously defined for the two carrier proteins in the central nervous system (18, 21). No immunoreactivity for either SAT1 or SAT2 was detectable in cell extracts from pure astrocytic cultures (data not shown) (18, 21).

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**Fig. 1. Western blot analysis of total tissue and cell homogenates stained with the anti-SAT1 and anti-SAT2 antibodies.** An anti-SAT1 and anti-SAT2 antibodies recognize bands of approximately 55 kDa in total homogenates of rat hippocampus and of 12-day-old hippocampal neurons.

Inc. (West Grove, PA). MeAIB and dimethylbenzamil were purchased from Sigma.

Statistical Analysis—Results are presented as means ± S.E. Data were statistically compared using the Student's t test. Differences were considered significant should p < 0.05.

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munoreactivity was present up to the leading edge of the axonal growth cone (Fig. 2C) as visualized by antibodies against synaptobrevin/VAMP2 (Fig. 2D). SAT2 staining was undetectable in the distal part of the axon, including the growth cone (Fig. 2E, G, and H). In mature neuronal cultures, both SAT1 (Fig. 3A) and SAT2 (Fig. 3E) were expressed in the soma and in virtually all neuronal processes as revealed by double staining with antibodies directed against the somatodendritic marker MAP2 (Fig. 3, B and F). However, whereas SAT1 immunoreactivity showed a clustered appearance (Fig. 3, A and C), SAT2 staining appeared to be more uniformly diffused in neuronal processes (Fig. 3, E and G). In synaptically connected neurons, SAT1 immunoreactivity, although present at the highest extent in neuronal cell bodies and dendrites, could also be detected at sites of synaptic contacts (Fig. 3C), which appeared as synaptobrevin/VAMP-positive puncta (Fig. 3D, arrows) outlining the neuronal cell body and dendrites. On the contrary, SAT2 staining was undetectable at synapses (Fig. 3, G and H, arrows). When hippocampal neurons were grown on top of a monolayer of astrocytes, SAT1 immunoreactivity, although present at the highest extent in neuronal cell bodies and dendrites, could also be detected at sites of synaptic contacts (Fig. 3C), which appeared as synaptobrevin/VAMP-positive puncta (Fig. 3D, arrows) outlining the neuronal cell body and dendrites. On the contrary, SAT2 staining was undetectable at synapses (Fig. 3, G and H, arrows). When hippocampal neurons were grown on top of a monolayer of astrocytes, SAT1 (Fig. 3F) and SAT2 (Fig. 3G) immunoreactivities showed a similar pattern of distribution being detectable in the cell body and processes of neuronal cells. No staining was detectable in astrocytes identified by the labeling with antibodies against the glial marker glial fibrillar acidic protein (Fig. 3, L and N). Inclusion of the glutathione-S-transferase fusion protein used to generate the antibodies abolished neuronal immunostaining (data not shown).

Functional Relevance of Glutamine Transport at the Glutamatergic Synapse—Astrocytes are known to enhance synaptic efficacy (30). In agreement with these data, significantly higher mEPSC amplitude (Fig. 4D) and frequency (Fig. 4C) were recorded from neurons growing on top of glial cells (Fig. 4B) as compared with neurons growing without glia (Fig. 4A) (mEPSC amplitude with glia: 1.46 ± 0.12, values normalized to neurons without glia (see “Experimental Procedures”) (n = 13 with glia; n = 16 without glia; p < 0.05) (Fig. 4F). Absolute values of mEPSC amplitude: 17.2 ± 8.4 pA, neurons without glia, and 25.15 ± 16.8 pA, neurons with glia (p < 0.05). mEPSC frequency with glia: 1.48 ± 0.14, values normalized to neurons without glia (n = 13 with glia; n = 16 without glia; p < 0.05) (Fig. 4E). Absolute values of mEPSC frequency: 2.29 ± 0.55 Hz, neurons without glia, and 2.32 ± 0.82 Hz, neurons with glia (p > 0.1). Note that when data, instead of being compared in the same neuronal preparation with or without glia (see “Experimental Procedures”), were analyzed by pooling responses together, no significant difference could be detected in mEPSC frequency because of the well known considerable variability of mEPSC frequency among different neuronal cultures. To investigate whether this increased synaptic efficacy could be at least partially produced by glutamine or alanine supplied by glial cells as glutamate precursors, we pharmacologically interfered with the neutral amino acid uptake by neuronal cells. A characteristic feature of system A, which differentiates it from systems L and ASC, is the ability to transport N-methylated substrates such as MeAIB, which competes for uptake. MeAIB used at concentrations previously shown to inhibit glutamine uptake by neurons (10–40 mM) (31) produced a significant reduction of mEPSC amplitude in neurons growing on monolayers of glial cells (Fig. 5, D–F). The effect was maximal 5–10 min after MeAIB perfusion (Fig. 5D) (mEPSC amplitude 5–10 min after 40 mM MeAIB: 0.78 ± 0.06, normalized to control values (n = 7; p < 0.001)). No changes in mEPSC amplitude were produced by MeAIB applied to hippocampal neurons growing in the absence of glial cells (Fig. 5, A–C) (mEPSC amplitude 5–10 min after 40 mM MeAIB: 0.99 ± 0.06, normalized to controls (n = 5; p > 0.5)). Furthermore, MeAIB did not alter neuronal responsivity to 20 μM glutamate measured as total current in whole-cell recordings (1.01 ± 0.18, values normalized to controls (n = 11; p > 0.5)) or as calcium response in fura-2-loaded neurons (0.97 ± 0.04, values normalized to controls (n = 6; p > 0.5)), thus excluding a postsynaptic effect of the inhibitor.

It has been recently demonstrated that reduced vesicular filling of glutamate into synaptic vesicles produces a reduction in mEPSC amplitude, also decreasing mEPSC frequency without altering synaptic vesicle recycling (32). In apparent contrast, MeAIB application was found to increase mEPSC frequency both in neurons growing on top of glial cells (Fig. 6, B and D) (mEPSC frequency 5–10 min after 40 mM MeAIB: 1.85 ± 0.33, normalized to controls (n = 7; p < 0.05)) and in neurons devoid of neighboring astrocytes (Fig. 6, A and C) (mEPSC frequency 5–10 min after 40 mM MeAIB: 2.38 ± 0.48,
normalized to controls (n = 5; p < 0.05)). As system A carriers transport MeAIB by symporting Na+/H+ ions (18, 21), we investigated whether the increase in mEPSC frequency could be produced by a cytosolic calcium increase consequent to the extrusion of Na+/H+ ions mediated by the Na+/Ca2+ exchanger as described previously (33). To address this point, mEPSC frequency and amplitude were measured upon MeAIB treatment in neuronal cultures exposed to 35 μM dimethylbenzamil, a blocker with a relatively high affinity for the Na+/Ca2+ exchanger (33, 34). Dimethylbenzamil was incubated in the bath 10–15 min before MeAIB application. Dimethylbenzamil did not significantly change mEPSC amplitude and frequency either in pure neuronal cultures or in neurons growing on top of glial cells (mEPSC amplitude in pure neuronal cultures: 17.2 ± 0.84 pA, control (n = 16); 16.96 ± 1.09 pA, dimethylbenzamil (n = 5; p > 0.1); mEPSC frequency in pure neuronal cultures: 2.29 ± 0.55 Hz, control (n = 16); 3.7 ± 0.77 Hz, dimethylbenzamil (n = 5; p > 0.1); mEPSC amplitude in neurons with glia: 25.15 ± 1.68 pA, control (n = 13); 24.27 ± 3.85 pA, dimethylbenzamil (n = 6; p > 0.1); mEPSC frequency in neurons with glia: 2.32 ± 0.82 Hz, control (n = 13); 2.05 ± 0.88 Hz, dimethylbenzamil (n = 6; p > 0.1)) (33). This treatment largely prevented the increase in mEPSC frequency consequent to MeAIB. In neurons devoid of glial cells in the presence of dimethylbenzamil, mEPSC frequency and amplitude were not significantly changed by MeAIB treatment (Fig. 6, E and G) (mEPSC frequency: 0.86 ± 0.34, normalized to controls (n = 5; p > 0.1); mEPSC amplitude: 0.93 ± 0.07, normalized to controls (n = 4; p > 0.1)). In neurons growing on top of glial cells and recorded in the presence of dimethylbenzamil, the MeAIB-dependent reduction in mEPSC amplitude was accompanied by a significant decrease in mEPSC frequency (Fig. 6, F and H) (mEPSC frequency: 0.32 ± 0.08, normalized to controls (n = 6; p < 0.05); mEPSC amplitude: 0.62 ± 0.08, normalized to controls (n = 5; p < 0.05)). In a percentage of treated cells (24%), dimethylbenzamil was found to be not effective.

**DISCUSSION**

The metabolic dependence of glutamatergic neurons upon glia via glutamine and alanine supply to provide the precursor for the neurotransmitter glutamate is widely established. The removal of glutamate from the synaptic cleft by astrocytic carriers (5, 6, 35), its conversion to glutamine by the enzyme glutamine synthetase (1, 2, 7, 36, 37), and the back conversion of glutamine to glutamate by a neuronal transaminase (38) are collectively referred to as "the glutamate-glutamine cycle." The recent identification of SAT1 as the first member of the system A family of neutral amino acid transporters, specifically localized on neuronal cells and endowed with a high efficiency in glutamine transport, has provided a missing link in the glutamate-glutamine cycle (18). The supply of α-ketoglutarate and alanine from glial cells also contributes to the neurotransmit-
ter synthesis through the conversion to glutamate via an aminoxyacetic acid-inhibitable transamination reaction (11, 12). The expression of a second member of the system A family, SAT2, in neurons has revealed a second possible gateway for astrocyte-derived glutamate precursors (21).

We have investigated the expression, subcellular distribution, and functional role of SAT1 and SAT2 in cultured hippocampal neurons. Our results indicated a neuron-specific expression of these carriers from early developmental stages. In both developing and mature neurons, SAT1 and SAT2 were present in all neuronal compartments including axons. However, whereas SAT2 staining was uniformly distributed in neuronal processes, SAT1 immunoreactivity had a slightly clustered appearance as described previously for the glutamate transporter excitatory amino acid carrier 1 (28). SAT1-positive hotspots may represent surface clusters of transporters and/or intracellular pools of the protein. The existence of intracellular pools of carriers that has been previously demonstrated for other neurotransmitter transporters (28, 39, 40) might open the possibility of a regulated recruitment of these carriers to the neuronal surface. Interestingly, only SAT1 was found to be present in the leading edge of axonal growth cones and at later developmental stages could be detected at the sites of synaptic contacts. In this respect, SAT2 and even more prominently SAT1 differ from the neuronal glutamate carrier excitatory amino acid carrier 1, which is excluded from the axon since early stages of neuronal development and is restricted to the dendritic shaft and spine neck in fully differentiated neurons (28). No significant difference in the carriers’ distribution was detected in neurons growing in contact with astrocytes when compared with neurons growing in astrocyte-conditioned medium.

The presence of system A carriers in glutamatergic neurons and in particular the localization of SAT1 at sites of synaptic contacts provide a strategic anatomical basis for a functional role of these transporters in synaptic function. In agreement with this possibility, the specific inhibitor of neutral amino acid transport system A, MeAIB, was found to reduce mEPSC amplitude in hippocampal neurons growing on top of astrocytes. Because no difference in receptor responsivity to glutamate was detected in the presence of MeAIB, we propose that the MeAIB-mediated reduction in mEPSC amplitude is consequent to a diminished amount of glutamate in synaptic vesicles produced by the inhibition of the astrocyte-mediated feeding of glutamine and/or alanine. Accordingly with this possibility, mEPSC amplitude was significantly higher in neurons growing on top of glial cells as compared with pure neuronal cultures. Furthermore, MeAIB specifically reduced mEPSC amplitude in neurons growing on top of astrocytes, being ineffective in neurons devoid of glial cells. Although MeAIB treatment does not

**FIG. 4. Enhanced mEPSC amplitude and frequency in hippocampal neurons growing on top of astrocytes.** Pure neuronal cultures (A) and astrocyte-neuron co-cultures (B) stained with antibodies directed against β-tubulin. C and D, representative recordings from 10–12-day-old hippocampal neurons growing either in the absence (C) or in the presence (D) of glial cells. E and F, histograms showing mEPSC frequency (E) and amplitude (F) in neurons co-cultured with astrocytes versus control neurons. Calibration bar: 40 μm (A and B).

**FIG. 5. The system A inhibitor MeAIB reduces mEPSC amplitude.** A and D, time course of the changes in the amplitude of mEPSCs recorded from either pure neuronal cultures (A) or from neurons co-cultured with astrocytes (D) upon the application of MeAIB (arrows). B and E, superimposed averaged mEPSC amplitude measured at the beginning of the whole cell recording (solid line) and after 5–10 min from MeAIB application (dotted line). C and F, histograms showing mEPSC amplitude in MeAIB-treated neurons normalized to controls. Note the significant reduction in mEPSC amplitude selectively in neurons growing in the presence of astrocytes.
allow evaluation of the relative contribution of SAT1 and SAT2, the synaptic localization of SAT1 might suggest a more prominent relevance of this transporter. In support of this possibility, it has been shown in hippocampal preparations, glutamate derived from glutamine is preferentially secreted at glutamatergic synapses (41–43), and the relative affinity of SAT1 for glutamine is higher than that for SAT2 (18, 21). In addition, intracellular perfusion with glutamine of single neurons growing in microislands but not of neurons in multineuronal networks led to a significant enhancement in mEPSC amplitude. A reduction of glutamate content in synaptic vesicles has been shown to decrease not only the amplitude but also the frequency of mEPSCs (32). In apparent contrast, MeAIB treatment induced an increase in mEPSC frequency both in pure neuronal cultures and in neurons growing on top of glial cells. Because MeAIB is co-transported with Na+ ions, we hypothesize that the resulting Na+ load could drive Ca2+ influx through the Na+/Ca2+ exchanger. A similar process has been shown to occur during recovery from intracellular acidification when the activity of the Na+/H+ exchanger rises the intracellular Na+ concentration and leads to enhanced neurotransmitter release consequent to the Ca2+ influx driven by the Na+/Ca2+ exchanger (33). Our data support this possibility as the Na+/Ca2+ exchanger inhibitor dimethylbenzamil prevented the increase in mEPSC frequency in pure neuronal cultures. Interestingly, when the astrocyte-neuron co-cultures were preincubated with the Na+/Ca2+ exchanger inhibitor, a clear effect of MeAIB in reducing mEPSC frequency was unmasked, thus indicating that the block of glutamine transport in neurons associated with glial cells diminishes both mEPSC frequency and amplitude. These observations raise the possibility that during intense neuronal activity, the symport of Na+ ions together with glutamine and/or alanine through SAT1 or SAT2 transporters may directly contribute in driving the Ca2+ influx leading to an increase in mEPSC frequency.

Our data reveal a novel mechanism by which astrocytes may influence glutamatergic function. It is now widely established that astrocytes profoundly affect neuronal and synaptic function. Glial cells increase the number of mature functional synapses in central nervous system neurons (30) and also enhance synaptic efficacy by both postsynaptic and presynaptic mechanisms (44). Although astrocytes may directly influence the clustering and/or the functionality of glutamate receptors or even modulate the extent of vesicle recycling at the presynaptic terminal, our data indicate that the enhancement of synaptic efficacy may be at least partially achieved through the replenishment of glutamate into synaptic vesicles. Interestingly, it has been shown recently that reducing the glutamate content of synaptic vesicles results in the formation of partially filled vesicles, which undergo release with normal probability (32). These data have led to the conclusion that the mechanisms involved in controlling the release of synaptic vesicles are not dependent on the state of filling of synaptic vesicles themselves. The role of glial cells to directly influence the amount of presynaptic neurotransmitter glutamate is therefore of significant interest in normal and pathophysiological synaptic transmission in the central nervous system. Under this respect, it is notable that the frequency of spontaneous epileptiform discharges, induced in hippocampal slices by γ-aminobutyric acid antagonists, are strongly reduced by the block of glutamine and alanine uptake in neurons. Therefore, the neuron-astrocyte coupling could mediate through the restoration of the presynaptic glutamate pool a plastic form of bidirectional communication, eventually leading to an enhancement of synaptic transmission in the long term range.

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