Glutamate mediates several modes of neurotransmission in the central nervous system including recently discovered retrograde signaling from neuronal dendrites. We have previously identified the system N transporter SN1 as being responsible for glutamate efflux from astroglia and proposed a system A transporter (SAT) in subsequent transport of glutamine into neurons for neurotransmitter regeneration. Here, we demonstrate that SAT2 expression is primarily confined to glutamatergic neurons in many brain regions with SAT2 being predominantly targeted to the somatodendritic compartments in these neurons. SAT2 containing dendrites accumulate high levels of glutamine. Upon electrical stimulation in vivo and depolarization in vitro, glutamine is readily converted to glutamate in activated dendritic subsegments, suggesting that glutamine sustains release of the excitatory neurotransmitter via exocytosis from dendrites. The system A inhibitor MeAIB (3-[α-methylamino-iso-butyric acid) reduces neuronal uptake of glutamate with concomitant reduction in intracellular glutamate concentrations, indicating that SAT2-mediated glutamine uptake can be a prerequisite for the formation of glutamate. Furthermore, MeAIB inhibited retrograde signaling from pyramidal cells in layer 2/3 of the neocortex by suppressing inhibitory inputs from fast-spiking interneurons. In summary, we demonstrate that SAT2 maintains a key metabolic glutamate/glutamine balance underpinning retrograde signaling by dendritic release of the neurotransmitter glutamate.

Keywords: amino acid, glutamate-glutamine cycle, neurotransmitter release, SLC38, SNAT2, synaptic plasticity

Introduction

The amino acid glutamate doubles as a metabolite and the prime excitatory anterograde neurotransmitter in the central nervous system (CNS) (Fonnum 1984). Glutamate signaling occurs at a majority of synapses in the brain and is therefore intrinsic to complex higher brain functions, including cognition and learning. Conversely, dysfunction of glutamate signaling, and glutamate excitotoxicity are associated with a variety of neuropathological conditions (Olney 1991; Janjua et al. 1992; Parsons et al. 1998; Miyamoto et al. 2003). Recently, glutamate has been implicated in retrograde signaling at select synapse populations: vesicular release of this neurotransmitter from neocortical pyramidal cell dendrites acts on metabotropic glutamate receptors recruited to perisomatic terminals of fast-spiking, parvalbumin-containing basket cells (FS cells), thus providing negative feedback to presynaptic gamma-aminobutyric acid (GABA) release (Zilberter 2000; Harkany et al. 2004). At present, the concept that glutamate acts as a retrograde messenger still lacks unequivocal support. Particularly, it remains to be elucidated how the dendritic pool of glutamate is generated and replenished.

Although evidence exists for reuptake and reuse of released glutamate at presynaptic terminals, and for glucose and monocarboxylates serving as glutamate precursors, the general concept is that synaptically released glutamate is taken up by perisynaptic astroglia and becomes converted into glutamine. Glutamine is then recycled to the nerve terminal for regeneration of glutamate (Danbolt 2001; Albrecht et al. 2007). This hypothesis is supported by the specific targeting of glutamate transporters to glial processes (Chaudhry et al. 1995) that contain glutamine synthetase, the key enzyme catalyzing the formation of glutamate from glutamine, is correspondingly enriched in neurons (Aoki et al. 2002, 2003). Thus, SN1 releases glutamine from glial cells (Chaudhry et al. 2001) and is preferentially expressed on glial processes surrounding synapses (Boulland et al. 2002, 2003). Thus, SN1 is ideally positioned to supply neurons with this precursor to generate the neurotransmitter glutamate (Chaudhry, Reimer, et al. 2002). Phosphate-activated glutaminase (PAG), catalyzing the formation of glutamate from glutamine, is correspondingly enriched in neurons (Aoki et al. 1991; Laake et al. 1999). However, the identity of plasmalemmal transporter(s) participating in glutamine uptake at nerve endings and/or dendrites in glutamatergic neurons remains unknown.

The system A transporter SAT2 (also termed ATA2/SNAT2/SNAT2/SA1) shows considerable homology with SN1 (above 50% at the DNA level) (Reimer et al. 2000; Sugawara et al. 2000; Yao et al. 2000), but is functionally distinct: the lack of coupling to H+ translocation enables SAT2 to utilize both the electrical and the chemical gradients of Na+, thus creating steeper glutamine concentration gradients, as compared with SN1 (Chaudhry,
SAT2 is expressed in a variety of cell populations throughout the body and has been suggested to participate in the uptake of glutamine and other neutral amino acids to sustain cellular metabolic status (Armano et al. 2002; Gonzalez-Gonzalez et al. 2005; Melone et al. 2006). In the brain, in situ hybridization demonstrates SAT2 expression restricted to a subset of neurons (Reimer et al. 2000; Yao et al. 2000), however with as yet unknown neuron-specific functions.

Here, we have studied the precise cellular localization of the SAT2 protein in the brain. We demonstrate that SAT2 is selectively enriched in glutamatergic neurons and preferentially targeted to the somatodendritic compartment in glutamatergic cells, supporting a possible coupling to retrograde signaling pathways. Upon electrochemical activation, dendritic glutamate levels are sustained at the expense of glutamine, with this amino acid conversion being critically dependent on neuronal activity in vitro as well as in vivo. Notably, SAT2 is required to maintain quasi steady-state intraneuronal glutamate levels during neuronal activity. Electrophysiological studies revealed that functional integrity of SAT2-mediated glutamate transport into pyramidal cell dendrites underscores retrograde signaling at FS to pyramidal cell inhibitory terminals. Overall, our data demonstrate that SAT2-mediated glutamate transport through the plasmalemma helps maintain intracellular glutamate homeostasis during neuronal activity.

Materials and Methods

Animal Care

Ten male rats (Wistar, 200–300 g) were obtained from Scanbur BK for the SAT2 localization studies, while E18 rat fetuses (Sprague-Dawley) or newborn rat pups (Wistar) were used to generate primary cortical or hippocampal cultures, respectively. Electrophysiology studies were performed in parasagittal brain slices (300 μm thick) prepared from the somatosensory cortex of 13- to 16-day-old rats (Wistar). Animal handling was under veterinary supervision. Experimental designs adhered to European regulations and were approved by local authorities (University of Oslo, Oslo, Norway; Stockholms Norra Djurséstaka Nämnd, Stockholm, Sweden [N26/05 and N38/05]). Male Fischer 344 rats (mean age 15 months, n = 9) were used in the in vivo experiments and were maintained in accordance with guidelines established by the National Institute of Health.

Generation of Antibodies against SAT2

The N-terminal is the most divergent region among the family of amino acid transporters. Amino acid sequence between residues 1–54 of SAT2 (Reimer et al. 2000) was therefore chosen as a target to generate antibodies. This sequence fragment was PCR amplified and subcloned into a pGEX-3X vector, C-terminal to the sequence for glutathione-S-transferase (GST). The fusion protein was induced in Escherichia coli by isopropyl-β-D-thiogalactopyranosid. The protein was purified on glutathione sepharose beads and directly used to immunize 3 rabbits. The ensuing antibodies, designated as SAT2-N1, SAT2-N2, and SAT2-N3, were affinity-purified as described in Danbolt et al. (1998) and Boulland et al. (2002). Briefly, sera were absorbed against immobilized GST on a Sepharose column (to remove unspecific antibodies), followed by isolation of specific antibodies by absorption onto immobilized GST fusion protein containing the N-terminal of SAT2. In addition, some of the sera were isolated using a GST fusion protein containing only the most divergent region of SAT2, spanning amino acid residues 23–54, to test for the consistency of our results. Indeed, identical staining was obtained with these latter antibodies.

Antibodies against Glutamate and Glutamine

The antibodies to glutamine and glutamate have been extensively characterized in previous studies (e.g., Ottersen et al. 1992; Laake et al. 1999). Their specificity and selectivity were tested by immunogold labeling in actual experiments, on conjugates of different amino acids that had been incorporated in resin sandwiches (Ottersen 1987) (Fig. S1B). The antibodies recognized their respective conjugates with high sensitivity and selectivity (insets, Fig. S1B), while only extremely low levels of immunogold labeling (<0.5%) were detected on the remaining conjugates. For information on commercial antibodies used in this study, see Supplemental Material.

Electrophoresis and Immunoblotting

Rat brains and COS7 cells transiently transfected with SAT2 were homogenized directly in sodium dodecyl sulfate (SDS) solubilization buffer containing 1% SDS, 1 mM phenylmethanesulfonyl fluoride, 5 mM ethylenediaminetetraacetate disodium salt, and 10 mM sodium phosphate buffer (NaPi, pH 7.4). The homogenates were run on 10% SDS-acrylamide gels, electroblotted onto nitrocellulose membranes, and immunostained with primary antibodies (0.1–0.2 μg/mL) over-night. Immunoreactivity was revealed by alkaline phosphatase- or horseradish peroxidase-conjugated secondary antibodies for qualitative analysis as described in Chaudhry et al. (1998) and Boulland et al. (2002).

Immunofluorescence Processing of Cell Cultures

Primary cortical cultures, made from E18 rat embryos as described in Oltedal et al. (2007), and PC12 cells transfected with empty pcDNA1amp vector or with pcDNA1amp containing HA (hemaglutinine epitope)-tagged SAT2 using Lipofectin 2000 (Invitrogen, Carlsbad, CA) and grown as described in Chaudhry et al. (1999) were immersion-fixed in 4% PFA (paraformaldehyde) in NaPi, (pH 7.4). Immunofluorescence single and double labeling of cultured cells was performed with SAT2-N1 (0.95 μg/mL) and additional markers as described in Chaudhry et al. (1999) and Oltedal et al. (2007).

Immunoperoxidase Processing of Rat Brain Sections

Male Wistar rats were transcardially perfused with 4% PFA in 0.1 M NaPi, and their brains were dissected out. Free-floating vibratome sections (50 μm) were cut and stained for SAT2 (applied at 9.5 μg/mL without Triton X-100 and at 0.95 μg/mL with 0.5% (v/v) Triton X-100) using the biotin-streptavidin-peroxidase system and 3,3'-diaminobenzidine as chromogen (Chaudhry et al. 1998). In simultaneous control experiments, primary antibodies were preincubated with 30 μg/mL of the fusion protein (aa 1–54) for 6 h. Anatomical nomenclature was adopted from the rat brain atlas of Paxinos and Watson (1998).

Electrical Stimulation and Monitoring of Glutamine/Glutamate Conversion In Vivo

Bilateral recording electrodes were implanted in the hilus of the area dentata and stimulating electrodes in the perforant pathway of adult rats as described in detail elsewhere (Barnes 1979; McNaughton et al. 1986). All rats had significant bilateral perforant path–granule cell evoked field potential responses (Supplementary Fig. S3). After a recovery period of 2 months, rats were deeply anesthetized with sodium pentobarbital (80 mg/kg), and then connected to the amplifiers and stimulators so that the responses could be reliably monitored throughout the procedure. The chest cavity was opened, the descending aorta clamped, and then a continuous 50-Hz stimulation was delivered to one of the hemispheres for 4.5 min prior to transcardial perfusion with 2.5% GA (glutaraldehyde)/1% PFA in 0.1 M NaPi. Subsequently, right and left hippocampi were dissected out, postfixed in the same fixative overnight, and changed into a storage solution of 10% diluted fixative in NaPi, therefrom.

Postembedding Immunogold Labeling of Brain Tissue

The medial part of the area dentata was dissected out, treated with 1% OsO₄ in 0.1 M NaPi, dehydrated in graded ethanol and propylene oxide, and embedded in Durcupan ACM. Ultrathin sections (<100 nm) were cut and mounted on nickel grids. Immunogold staining was done as described in Chaudhry et al. (1998).
Quantification and Statistical Analysis of Immunogold-Labeled Sections from Electrically Stimulated Rat Brains

Subsequent morphometric analyses were performed by experimenters who were blind to prior procedures. One rat was discarded due to poor preservation of the hippocampal tissue. Electron micrographs were viewed on a Philips CM10 transmission electron microscope. Images were taken at random in the middle and inner molecular layers (MML and IML) of area dentata, from the stimulated and unstimulated hemisphere. Image acquisition and analysis were carried out by 2 different persons according to 2 different procedures.

Procedure 1
Images from 4 animals were recorded on 36 mm films at a primary magnification of ×6500 and printed at a final magnification of ×48 000. Regions of interest (ROIs) were marked and measured with a digitizing table and the number and density of gold particles calculated by the computer program MORFOREL as described in Blackstad et al. (1990).

Procedure 2
Images from 6 animals were recorded with a MegaView II CDD camera driven by the image processing software AnalySIS as described in Mathiesen et al. (2006). See Supplemental material for details.

Hippocampal Neuronal Cultures and Pharmacological Analyses
Primary mixed hippocampal cultures from CA1-CA3 regions of 1–4 days old rats were grown in a medium containing glutamine and depolarized by means of the KCl method as described in Olteanu et al. (2007) (see supplemental material for further procedural details). Stimulation was terminated instantaneously by replacing the medium with 2.5% GA and supplemental material for further procedural details). Stimulation was terminated instantaneously by replacing the medium with 2.5% GA and supplemental material for further procedural details).

Images from 6 animals were recorded with a MegaView II CDD camera driven by the image processing software AnalySIS as described in Mathiesen et al. (2006). See Supplemental material for details.

Cortical Cultures and Determination of Glutamine Metabolites
Primary cortical cultures were prepared from E18 Sprague-Dawley rat embryos as described in Berghuis et al. (2006) and cultured for 7 days. Subsequently, n = 3 cultures (24-well inserts with 200 000 cells/insert) per condition were exposed to: 2 mM Gln-containing DMEM/F12 medium (control), DMEM/F12 supplemented by an additional 3 mM Gln (total [Gln] = 5 mM), and 2 mM Gln-containing culture medium with MeAIB (5 mM; MeAIB, Sigma) for 2 or 6 h. In parallel, identical cultures were treated as above with KCl (20 mM) as additive to evoke sustained depolarization of cortical neurons. After incubation, culture media (400 μL) were collected and snap-frozen for high-performance liquid chromatography (HPLC) analysis. In addition, cells were scraped into 0.05 M perchloric acid (100 μL per sample), lyzed at 4 °C for 60 min, and stored at −80 °C until analysis to determine intracellular amino acid concentrations after neutralization with 0.05 M NaOH. Intracellular and extracellular amino acid concentrations were determined after pre-column derivatization by isocratic HPLC analysis of ortho-phthalaldehyde derivatives of glutamate, aspartate, and glutamine as previously described (Harkany et al. 2000). Statistical analysis was performed by first testing differences between particular experimental groups (Student’s t test) followed by a multivariate ANOVA design with [Gln], [KCl], and incubation time used as fixed variables (SPSS; Supplementary Table 1).

Dual Patch-Clamp Electrophysiology
Rat pups were sacrificed by cervical dislocation under deep isoﬂuorane anesthesia. In parasagittal slices spanning the somatosensory cortex (layer 2/3), FS cells and pyramidal cells were identiﬁed by infrared differential interference contrast video microscopy and subsequently analyzed for neuronal firing properties (Harkany et al. 2004). Simultaneous dual whole-cell current-clamp recordings were made in pyramidal cells synaptically connected to FS cells using Axoclamp 200B and Axoclamp 2B amplifiers (Axon Instruments, Foster City, CA). See Supplemental Material for details. The average resting membrane potential of pyramidal cells was −80 ± 0.9 mV and the average input resistance was 190 ± 0.03 MΩ. These values are comparable to previous reports on pyramidal cells incubated without KCl (−77 mV and 197 MΩ; Zilberter et al. 1999; Holmgren et al. 2003). Dendritic glutamate pools of neocortical pyramidal cells were substantially depleted by sequential incubation of cortical slices in an extracellular solution containing 20 mM KCl and 10 μM MeAIB for 60 min with their subsequent transfer into extracellular solution supplemented by 10 μM MeAIB throughout the recordings. In control experiments, slices were exposed to 20 mM KCl for the above period with subsequent transfer into normal extracellular solution. All experiments were performed at 32–34 °C with continuous oxygenation of the solutions. Changes in short-term synaptic plasticity at inhibitory synapses of FS cells innervating neocortical pyramids was tested by using published protocols (Zilberter 2000; Holmgren et al. 2003; Harkany et al. 2004). In brief, a train of 10 backpropagating action potentials (bAPs) at 50 Hz in a postsynaptic pyramidal cell was followed by a single inhibitory postsynaptic potential (IPSP) evoked by somatic stimulation of the FS cell 250 ms later. This pattern of stimulation was repeated at a frequency of 0.14 Hz for a total of 85 sweeps. Data on changes of synaptic efficacy are presented as mean ± SEM. The probability of IPSP suppression (deviation from the average IPSP amplitude defined as 100% in control) was tested using Student’s t test.

Results

Characterization of the Novel SAT2 Antibodies
Because the precise subcellular localization of a given protein, such as SN1, can be indicative of its putative function(s) (Boulland et al. 2002; Solbu et al. 2005), we generated antibodies specific against SAT2 to determine its cellular and subcellular localization in the adult rat brain. In general terms, the N-terminal is the most divergent region amongst members of the SLC38 family of amino acid transporters which include SAT2 (Chaudhry, Schmitz, et al. 2002). In addition, the 1–54 amino acid sequence of SAT2 contains amino acid residues with high antigenicity (Reimer et al. 2002; Solbu et al. 2005), we generated antibodies specific against SAT2 to determine its cellular and subcellular localization in the adult rat brain. In general terms, the N-terminal is the most divergent region amongst members of the SLC38 family of amino acid transporters which include SAT2 (Chaudhry, Schmitz, et al. 2002). In addition, the 1–54 amino acid sequence of SAT2 contains amino acid residues with high antigenicity (Reimer et al. 2002) and was therefore chosen as a target to generate antibodies. Three rabbits were immunized with a GST fusion protein containing these initial 54 amino acids of SAT2 and the sera obtained were affinity-puriﬁed by different procedures to secure specificity (see Material and Methods for details).

Extracts from COS7 cells transiently expressing SAT2 or an empty vector were subjected to SDS-PAGE (polyacrylamide gel electrophoresis) and immunolabeled with afﬁnity-puriﬁed SAT2 antibodies. SAT2 expression was detected by all 3 antibodies (a representative experiment with SAT2-N1 is shown in Fig. 1A, lane 1), appearing as a smear, as reported for other transiently expressed transporter proteins in mammalian cells (Peter et al. 1995; Boulland et al. 2002). Only a weak band at about 100 kDa, was detected on immunoblots of COS7 cell extracts expressing an empty vector (Fig. 1A, lane 2). SDS extracts of the adult rat brain were also electrophoretically separated, stained with SAT2-N1, -N2, and -N3, and the reaction visualized by alkaline phosphatase. A broad band, as is typical of plasma membrane transporter proteins (Dehnes et al. 1998; Boulland et al. 2002), was detected at around 60 kDa by all 3 antibodies (Fig. 1B, lanes 1–3), which is close to the predicted theoretical value of ~55 kDa for this protein (Reimer et al. 2000). Increasing the protein concentration of brain lysates from 2 to 30 μg did not reveal any
Figure 1. Characterization of SAT2 antibodies. (A) Three rabbits were immunized with a fusion protein containing the initial 54 amino acids of the N-terminal of SAT2. The sera were affinity-purified and the antibodies were tested for specificity on immunoblots of electrophoretically separated (SDS-PAGE) extracts from COS7 cells transfected with SAT2 or an empty vector. A strong diffuse smear was detected by the SAT2-N1 antibody on extracts from cells transfected with SAT2 (1). Only a faint band around 100 kDa was seen on extracts from COS7 cells transfected with the empty vector (2). (B) The affinity-purified antibodies against SAT2 were applied on electrophoretically separated whole brain extracts and the immunoreaction visualized by alkaline phosphatase conjugated secondary antibodies. A single broad band was detected at around 60 kDa for all 3 antibodies SAT1-N1-3 (1-3). No band was detected when the primary antibody was omitted (4). (C) No additional bands were detected upon increasing the amount of protein loaded on the gel from 2 to 30 μg, stained by SAT2-N1 and visualized by chemiluminescence. (D, E) Primary cortical cultures were colabeled with SAT2-N1 antibody (green) and select markers for different cell types (red). SAT2 does not colocalize with GAD65 (glutamic acid decarboxylase), a marker for GABAergic interneurons, or with GFAP (glial fibrillary acidic protein), a marker for astrocytes. Scale bars: 20 μm (D, E).

Figure 2. Differential subcellular localization of SAT2 in select populations of neurons in the CNS. (A) Immunoperoxidase staining of parasagittal brain sections by SAT2-N1 reveals SAT2 immunoreactivity in the olfactory bulb (OB), cortex (Cx), hippocampus (Hc), thalamus (Th), cerebellum (Cb), colliculus inferior (Cl), and the dorsal cochlear nucleus (DCN). Low levels of SAT2 immunoreactivity are detected in caudoputamen (GPu), globus pallidus (Gp), and substantia nigra (SN). (B) Only weak, diffuse staining of the molecular layer of the cerebellum remains upon preincubation of the antibodies with the GST fusion protein containing the N-terminal of SAT2 used to immunize the rabbits. (C) SAT2 is expressed at high levels in the choroid plexus (PC). (D) No staining of the choroid plexus is detected when applying SAT2-specific antibodies preincubated with the fusion protein containing the N-terminal of SAT2. (E, * in A) In the thalamus, strong SAT2 staining is detected in neuronal perikarya and processes. (F) In the brain stem, strong SAT2 immunosignal is observed in cell bodies of principal cells of the mesencephalic trigeminal nucleus. (G, square in F) Note strongly stained evaginations at the periphery of perikarya in this nucleus. Only weak staining of a subset of processes is seen in the caudoputamen (H) and in the substantia nigra (I). Scale bars: 250 μm (A, B), 50 μm (C, D), 500 μm (F). 25 μm (E, G, H, I).

Differential Cellular and Regional Localization of SAT2 Suggests Transporter Functions Associated with Glutamatergic Neurotransmission

In primary cortical cultures, SAT2 staining appeared in neuron-like cells. Double-labeling experiments for SAT2 and additional cell-type specific phenotypic markers, revealed colocalization with the somatodendritic marker MAP2 (microtubule associated protein-2A and B; data not shown), but not with GAD65 (Fig. 1D), the enzyme converting glutamate to GABA specifically expressed by GABAergic neurons (Mugnaini and Oertel 1985), or with glial fibrillary acidic protein (GFAP) (Fig. 1E) expressed in astroglia (Eng et al. 2000). Thus, our study demonstrates preferential SAT2 expression in glutamatergic neurons in vitro.

The regional distribution of SAT2-expressing cells in the CNS was studied by immunoperoxidase staining of free-floating parasagittal sections of adult rat brains. Survey of the sections by low-power light microscopy revealed strong SAT2 staining in several brain regions, including the hippocampus, neocortex, olfactory bulb, cerebellum, thalamus and the dorsal cochlear nucleus (Fig. 2A). In contrast, low levels of SAT2 staining were detected in the striatum, globus pallidus, and substantia nigra pars reticulata, harboring significant densities of GABAergic perikarya and nerve endings (Mugnaini and Oertel 1985).
High-resolution light microscopy also revealed prominent SAT2 staining in the choroid plexus (Fig. 2C), which was abolished upon preincubation of the antibody with the fusion protein used for its generation (Fig. 2D). In the brain parenchyma, strong SAT2 immunoreactivity was detected in scattered cells, for example, in the ventrobasal thalamic complex (Fig. 2E). These cells with multipolar perikarya and large size represent the relay neurons, which establish the glutamatergic thalamocortical projection (Spreafico et al. 1983). Similar to our findings in primary cortical cultures (Fig. 1f), SAT2 was found both in cell bodies and processes (Fig. 2E). In the brain stem, strong SAT2 immunoreactivity occurred in the characteristic principal cells of the mesencephalic trigeminal nucleus (Fig. 2F,G). These large cells have perisomatic evaginations which mediate direct contact with axon terminals, and are known to express PAG and display strong glutamate immunoreactivity (Chen et al. 2001; Lazarov 2002). SAT2 staining outlined the cell bodies, and occurred in particular in the spinous projections (Fig. 2G).

The caudatoputamen contains cholinergic interneurons intermingled with GABAergic long-range projection neurons terminating in the globus pallidus and substantia nigra (Ribak et al. 1976; Chaudhry et al. 1998). Striatal territories receive massive glutamatergic input from the cerebral cortex but contain few if any glutamatergic perikarya. Accordingly, no SAT2-stained cell bodies or terminals were detected in the caudatoputamen. Only weak staining of some irregular processes was observed (Fig. 2H). Similar weak staining of processes was seen in the substantia nigra pars reticulata (Fig. 2I), which also predominantly contains GABAergic neurons (Ribak et al. 1976). In addition, SAT2 immunoreactive cells were not seen in the substantia nigra pars compacta that harbors dopaminergic perikarya (data not shown). Thus, SAT2 immunoreactivity shows subcellular and regional variations in the adult rat brain with a selective enrichment in areas harboring glutamatergic neurons.

**SAT2 is Enriched in Select Glutamatergic Neuron Populations**

Strong SAT2 staining was present in layer V of the neocortex (Fig. 3A). Here, SAT2 labeling occurred in the cell bodies of large, often pyramidal-shaped cells (Fig. 3A') and extended throughout their stout radially oriented dendrites ramifying into a bouquet in the superficial cortical layer (Fig. 3A'). The shape of the cells and their localization are suggestive of typical glutamatergic cortical pyramidal cells (Ottersen and Storm-Mathisen 1984; Conti et al. 1989; Nieuwenhuys 1994). This somatodendritic SAT2 immunoreactivity confirmed the results from primary cortical cultures (Fig. 1E). Weaker SAT2 staining was detected in some smaller cells—likely to represent interneurons—concentrated around pyramidal cells in layer V and in superficial layers (Fig. 3A, arrow). Thus, our data suggest differential SAT2 expression by neocortical pyramidal cells and putative interneurons.

In the olfactory bulb, SAT2 was strongly expressed in the external plexiform layer (Fig. 3B). This layer contains dendrites of mitral, tufted and granule cells (Ottersen and Storm-Mathisen 1984). Dendro-dendritic reciprocal synapses are responsible for neuronal synchronization by release of glutamate from the mitral cells and reciprocal inhibitory action from the granule cells in this layer (Didier et al. 2001). We detected strong SAT2 staining in a majority of the processes, most likely dendrites, of the external plexiform layer. Strong staining was also detected in the somata of mitral cells, recognized as glutamatergic neurons (Nakanishi 1995) (Fig. 3B'). In contrast, only weak labeling occurred in the cell bodies of granule cells (Fig. 3B', arrow), which are GABAergic neurons (Nakanishi 1995). In the glomeruli, SAT2 staining appeared in some processes extending from the external plexiform layer (Fig. 3B'). Recently, it has been reported that subsets of juxta-glomerular cells make interglomerular excitatory connections and are responsible for the center-surround inhibition among olfactory bulb glomeruli (Aungst et al. 2003). We detected scattered SAT2 stained juxta-glomerular cells (Fig. 3B', arrow).

The cerebellum has a high density of glutamatergic cells, i.e. the granule cells, but also contains GABAergic Purkinje cells and GABAergic interneurons. Some staining was detected in the dendrites of Purkinje cells (data not shown; cf. Fig. 2A). However, low levels of staining remained in these dendrites upon preincubation of the antibodies with the immunizing fusion protein (Fig. 2H) thereby suggesting the nonspecific contribution of IgG immunostaining, which is common in these dendrites (Yoshimi et al. 2002). In addition, no mRNA for SAT2 has been reported in Purkinje cells by in situ hybridization.
Only weak staining was found in GABAergic Golgi cells in the granule cell layer and stellate and basket cells in the molecular layer, and in Bergmann fibers (data not shown). In contrast, we found very strong staining in some scattered cells in the granule cell layer. The large nucleus, stout dendrite (Fig. 3C, arrow) and their preferential localization to the vestibulocerebellum strongly suggest SAT2 expression in putative glutamatergic unipolar brush cells (UBCs) (Mugnaini et al. 1997). The staining of glutamatergic granule cells appears weaker (Fig. 3C, arrow), which is to be expected due to the scarcity of their cytoplasm. In the cerebellar nuclei, the majority of neurons are glutamatergic (Batini et al. 1992). These cells are the principal projection neurons carrying information from the cerebellum to other brain regions (Giuffrida et al. 1993; Schwarz and Schmitz 1997). Coincidentally, our data show strong SAT2 staining in cerebellar nuclei (Figs 2A, 3D). Thus, similar to the neocortex and the olfactory bulb, SAT2 expression is enriched in somata and dendrites of glutamatergic cells in the cerebellum.

**SAT2 Immunoreactivity is Pronounced in Glutamatergic Neurons in the Hippocampus**

In the hippocampus, moderate to strong SAT2 staining was seen in glutamatergic principal neurons: pyramidal cells of the CA1–3 subfields and granule cells of the dentate gyrus (Fig. 4A). Antibody specificity for SAT2 was demonstrated throughout this brain region by abolishing the staining pattern upon preincubating the antibodies with the fusion protein containing the targeted amino acid sequence of SAT2 (Fig. 4B). SAT2 staining was pronounced both in the cell bodies and in the dendrites of principal cells (Fig. 4C,D). SAT2 staining appeared strongest in superficially located granule cells, which are the earliest to be formed and therefore most mature (Piatti et al. 2006). Immunogold labeling of ultrathin sections revealed high levels of glutamine in the somatodendritic compartments of.

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**Figure 4.** SAT2 is restricted to hippocampal glutamatergic neurons containing high levels of glutamine. (A) Immunoperoxidase staining of the hippocampus with affinity-purified antibodies towards SAT2, shows staining in the granule cell and pyramidal cell layers. (B) Such staining is abolished upon preincubation of the antibodies with the fusion protein used for immunization. (C) High power light microscopy shows SAT2 labeling of the cell bodies as well as the dendrites of pyramidal cells in the CA1. (D) In the dentate gyrus, SAT2 staining occurs in the cell bodies and dendrites of the granule cells, particularly strongly in the superficial part of the granule cell layer. (E) Scattered SAT2 stained cells, resembling mossy cells, are detected in the hilus. (F) In the stratum radiatum, axon bundles (arrow) are seen crossing dendrites (arrow heads) of CA3 pyramidal cells. (G) Immunogold electron microscopy with antibodies directed to glutamine shows accumulation of glutamine in the SAT2 expressing dendrites of pyramidal cells in the CA1 (cf. C). Some adjacent terminals (T) have comparatively less glutamine. D, dendrite; G, granule cell layer; H, hilus; LM, stratum lacunosum-moleculare; M, molecular layer; O, stratum oriens; P, pyramidal cell layer; R, stratum radiatum; SL, stratum lucidum; T, nerve terminal. Scale bars: 500 μm (A and B) and 20 μm (C–F).
these neurons (Fig. 4G). No SAT2 stained terminal-like structures were seen in the molecular layer of the dentate gyrus. This region receives the nerve terminals of the perforant path from the area entorhinalis (outer two thirds) and of the mossy cells in the hilus (inner one third) (Witter and Groenewegen 1984). Similarly, no staining was detected in terminal-like structures in the stratum radiatum and stratum oriens of, for example, CA1, which are the targets of Schaffer collaterals and commissural fibers originating in the CA3 pyramidial cells. All of these nerve terminal systems are glutamatergic and are enriched in glutamate (Storm-Mathisen et al. 1983; Bramham et al. 1990; Malenka and Nicoll 1999), which becomes depleted on depolarization (Ottersen et al. 1990; Gundersen et al. 1998b). However, SAT2 staining is detected in fibers pervading the stratum lucidum where mossy fibers are located (Fig. 4F). These afferents originate in granule cells in the area dentata (Gaarskaer 1986) and exhibit a glutamatergic phenotype (Storm-Mathisen et al. 1983; Ottersen et al. 1990) but also contain some GABA (Ottersen and Storm-Mathisen 1986; Sandler and Smith 1991; Gutierrez 2005). Some interneuron-like cells were weakly stained in the stratum oriens, while no stained interneurons were detected in the molecular layer of area dentata, the stratum radiatum and the stratum lacunsum-moleculare (data not shown). These layers have high levels of GABA synthesis (Fonnum and Storm-Mathisen 1969) coincident with the presence of GABAergic afferents as well as preferential localization of various GABAergic interneuron subclasses (Mognain and Oertel 1985; Freund and Buzsaki 1996). Some cells in the hilus with 2 to 3 thick dendrites, resembling mossy cells, were also moderately to strongly immunoreactive for SAT2 (Fig. 4E). These calretinin expressing cells do not express GABA, but are the origin of most of the glutamatergic asymmetric synapses impinging on dendrites of granule cells in the inner third of the dentate molecular layer (Buckmaster et al. 1996; Blasco-Ibanez and Freund 1997).

To further establish the cellular and subcellular distribution of SAT2 in the hippocampus, we double-labeled brain sections with specific markers for different cell types and subcellular compartments. SAT2 colocalized with the neuronal somato-dendritic marker MAP2 in dentate granule cells (Fig. S1C) corroborating our immunoperoxidase staining pattern (Fig. 4D). We did not observe any colocalization of SAT2 with neurofilaments in axons invading the granule cell layer (Fig. S1D), arguing against a role of SAT2 in GABAergic interneurons involved in the regulation of granule cell synchrony (Freund 2003). No colocalization was seen of SAT2 and the astroglial marker GFAP (Fig. S1E) (except weak staining with one of the 3 SAT2 antibodies, see above) or with myelin basic protein, an oligodendrocyte marker (Fig. S1F).

**High-Frequency Stimulation of the Perforant Path In Vivo Triggers Glutamate Accumulation at the Expense of Glutamine Availability in Nerve Terminals and Corresponding Subsynaptic Dendrites**

The pronounced expression of SAT2 in subsets of glutamatergic neurons argues for a role for SAT2 in glutamate neurotransmission. We hypothesized that SAT2 in glutamatergic neurons may mediate the uptake of glutamate for transmitter synthesis and that glutamine uptake may be rate limiting for the replenishment of released glutamate, in particular under depolarizing conditions. To test these possibilities in vivo, we stimulated the perforant path immediately before and during perfusion of animals with fixative (Fig. S3) and subsequently measured changes in the concentrations of these 2 amino acids in different neuronal compartments stimulated and unstimulated nerve terminals, and their postsynaptic dendrites. Quantitative electron microscopy of ultrathin sections revealed an essentially normal number of synaptic vesicles in the nerve endings of the perforant path that had been stimulated, compared with unstimulated contralateral nerve endings (6% reduction, $P = 0.38$, Student’s $t$-test). Next, the sections were single or double-labeled for electron-microscopic localization of glutamine and glutamate by the immunogold method. The stimulated perforant path projects to the MML of the area dentata. On the unstimulated side, we observed strong labeling for glutamine in terminals in the MML of area dentata (Fig. 5A). In contrast, on the stimulated side, the intensity of glutamine labeling in corresponding nerve terminals was reduced (Fig. 5B). This reduction is specific to the target area of the stimulated perforant path as glutamine immunoreactivity of nerve terminals in the IML (which does not contain perforant path terminals) did not differ from their counterparts on the unstimulated side (data not shown). Interestingly, double-labeling experiments for glutamate (15 nm particles) and glutamine (30 nm particles) showed a reduction in the concentration of glutamine, but not of glutamate in target dendrites in the stimulated compared with the unstimulated perforant path (Fig. 5C,D). This reduction was even more pronounced over mitochondria (the site of glutamine to glutamate conversion by PAG) in the dendrites postsynaptic to terminals of the stimulated perforant path (Fig. 5E,F). To test whether these changes occurred specifically in electrically activated structures, we measured, within the same section, the ratio of glutamine in the nerve terminals of the perforant path to glutamine in nerve terminals in the adjacent IML (i.e., derived from the mossy cells of the dentate hilus). A 30%, statistically significant ($P < 0.001$), reduction was detected in the stimulated hemisphere as compared with the unstimulated side (Fig. 5G,H). This observation suggests that the reduction specifically occurs in the nerve endings of the perforant path, that is, it cannot be ascribed to general changes in the tissue on the stimulated side. To further study the potential role of glutamine in the generation of glutamate in glutamatergic neurons in vivo, we tested the glutamate/glutamine ratio in the nerve terminals as well as in their postsynaptic dendritic elements in the middle and the inner thirds of the molecular layer on the stimulated and unstimulated hemispheres (Fig. 5I). A significant increase in the glutamate/glutamine ratio was seen specifically in the stimulated nerve endings of the MML. In contrast, negligible changes were seen in the glutamate/glutamine ratio in the nerve endings of mossy cells in the IML (no reduction in glutamine was seen in these terminals, but the glutamate labeling increased by 15%). An increased ratio between glutamate and glutamine labeling intensities was also detected in the dendrites of the synaptically stimulated granule cells (Fig. 5I) (glutamine concentration decreased by 30% in the nerve terminals in the MML, $P = 0.003$, and by 21% in the dendrites of the MML, $P = 0.05$). Interestingly, there was
a striking reduction in the glutamine concentration in mitochondria present in granule cell dendrites in the MML but not in the IML (Fig. 5f; cf. Fig. 5E,F). This points towards input selectivity as well as restricted metabolic changes in dendrite segments directly contacted by stimulated synapses. Collectively, our data suggest that the conversion of glutamine to glutamate is enhanced upon synaptic excitation in vivo in glutamatergic nerve endings and in subsynaptic dendrites of excitatory neurons also expressing SAT2. Moreover, these results indicate that glutamate is generated in the mitochondria prior to accumulation in secretory granules/vesicles.

**Glutamine Exposure Increases MEAIB-Sensitive Glutamate Production by Cortical Neurons under Depolarizing Conditions In Vitro**

To further assess whether glutamine accumulated in the somatodendritic compartment of SAT2-expressing neurons plays a role in the generation of glutamate, we determined temporal changes in glutamate and glutamine contents in dendrites of cultured pyramidal cells depolarized by 20–90 mM K+ (Gundersen et al. 1998b). We observed an initial slight increase in the concentration of glutamate in the dendrites of pyramidal cells after 1 min of depolarization, followed by a gradual Ca2+-dependent reduction (55 mM K+; Fig. 6A,B). These changes ubiquitously occurred when K+ was elevated, irrespective of its actual concentration in these experiments (data not shown). Glutamate levels showed an abrupt decline between 1 and 10 min after the onset of stimulation (Fig. 6C,D). These results lend support to the hypothesis that glutamine (Fig. 4G) can be recruited to the generation of neurotransmitter glutamate within pyramidal cell dendrites in an “on-demand,” activity-dependent fashion.

Given the concentration of SAT2 in dendrites of pyramidal cells both in the intact brain (Fig. 4C) and in primary cultures (Fig. 1E), it may contribute to the control of dendritic glutamate levels. To elucidate the functional significance of neuronal SAT2-mediated glutamate transport, we moderately depolarized primary cortical cultures by 20 mM K+ while
simultaneously exposing these cells, predominantly pyramidal neurons, to extracellular glutamine (2 or 5 mM in culture medium). In addition, we applied MeAIB, a nonmetabolized amino acid analog and a prototypic substrate of SATs to competitively disrupt the affinity of SAT2 for its natural substrates (Chaudhry, Schmitz, et al. 2002). Under control conditions, extracellular glutamine becomes depleted as a function of time (Fig. 7A). Parallel increases in the extracellular glutamate concentration indicate that glutamine is converted to glutamate (Fig. 7B; see Supplementary Table 1 for statistical details). Unchanged intracellular glutamate levels denote that cellular glutamine metabolism is tightly regulated both during unstimulated conditions (Fig. S2) and upon K⁺-induced membrane depolarization (Fig. 7C). Increased extracellular glutamate concentrations (5 mM) facilitate glutamate uptake in depolarized neurons (Fig. 7A) and result in an enhanced turnover as reflected by a progressive increase in glutamate released into the culture medium (Fig. 7B). The lack of change in intracellular glutamate concentrations validate that our experimental conditions did not perturb the intracellular control of glutamate metabolism (Fig. 7C).

MeAIB potently inhibited glutamate uptake by cortical neurons (Fig. 7A; Supplementary Table 1). Neuronal glutamate release into the culture media was unaltered upon MeAIB exposure (Fig. 7B). Nevertheless, this was achieved by utilizing intracellular glutamine stores (Fig. 7C). Without K⁺ treatment, the MeAIB-induced block of glutamate uptake resulted in a ~28% decrease in intracellular glutamate (Fig. S2). This was augmented to a ~41% reduction of the glutamate concentration by K⁺-induced membrane depolarization (Fig. 7C). The glutamate release observed in the presence of MeAIB (Fig. 7B) can be attributed, at least partly, to the fact that MeAIB through SATs per se depolarizes neurons (Chaudhry, Schmitz, et al. 2002). Other amino acids not released...
through quantal exocytosis, such as valine, did not show significant changes upon exposing the cultures to either glutamate or MeAIB (data not shown).

Compelling evidence suggests that glutamine serves as a precursor for aspartate. Aspartate may be coreleased with glutamate from excitatory nerve endings (Gundersen et al. 1998a). Elevated extracellular glutamine levels (up to 5 mM) did not significantly alter intracellular aspartate concentrations at basal K⁺ levels, and marginally counteracted depletion of intracellular aspartate levels upon K⁺-induced depolarization (see Fig. 7D vs. Fig. S2C). However, intracellular aspartate levels became progressively reduced in the presence of MeAIB implying the dependence of aspartate metabolism on system A-mediated glutamine transport (Fig. 7D; Supplementary Table 1).

**MeAIB Inhibits Retrograde Synaptic Signaling by Glutamate**

Previous studies have identified an involvement of endocannabinoid-independent retrograde signaling in inducing short-term synaptic depression at inhibitory synapses of nonaccommodating FS cells targeting the perisomatic segment and proximal dendrites of pyramidal cells in layer 2/3 of the neocortex (Zilberter 2000; Harkany et al. 2004) (Fig. 8A). This signaling mechanism is mediated by vesicular neurotransmitter release from pyramidal cell dendrites (Zilberter 2000). Vesicular glutamate transporter 3 (VGLUT3) accumulation at subsynaptic dendrites of neocortical pyramids (Harkany et al. 2004) together with the pharmacological augmentation of retrograde signaling by metabotropic glutamate receptor 1 (mGluR1) agonists and its occlusion by mGluR1/2 antagonists (Zilberter 2000; Zilberter et al. 2005) suggest that glutamate may act as retrograde messenger at these synapses. We hypothesized that retrograde signaling from pyramidal cell dendrites is reliant on SAT2-mediated replenishment of glutamate pools if glutamate is the retrograde messenger. Depletion of dendritic glutamate pools by MeAIB should thus diminish the efficacy of retrograde signaling, which would be reflected by a reduced IPSP suppression at unitary connections between FS and pyramidal cells. To deplete the physiological dendritic glutamate pool prior to recording synaptic efficacy in dual whole-cell current-clamp recordings, slices were transiently preincubated in an external solution containing 20 mM K⁺ and 10 µM MeAIB for 60 min. Subsequently, cortical slices were transferred into a bath and superfused by a solution containing 10 µM MeAIB throughout the recordings. In this paradigm, the increased extracellular K⁺ concentration served to deplete intracellular glutamate pools through sustained synaptic activity (Hamberger et al. 1979), while at the same time, MeAIB prevented the replenishment of intracellular glutamate by inhibiting SAT2 (Fig. 7C). To ensure that equal conditions were used for both MeAIB and control experiments, control slices were also preincubated in 20 mM K⁺-containing extracellular solution for 60 min. However, they were allowed to recover in normal superfusion solution afterward.

In control experiments, induction of retrograde signaling ("conditioning") by a preceding train of action potentials in the pyramidal cell resulted in a significant decrease of the IPSP amplitude (average gain 0.70 ± 0.02 of control, n = 5; P < 0.05; Fig. 8B,B’), in accordance with previous studies (Zilberter 2000; Harkany et al. 2004). These data indicate that prior exposure of neocortical slices to 20 mM K⁺ (for 60 min) does not significantly affect the degree of synaptic depression. Under SAT2 blockade, however, the result of the same conditioning protocol was a robust and sustained reduction of IPSP depression leading to substantial diminution (Fig. 8B”,B”’) of retrograde signaling (average gain of 0.95 ± 0.04 as calculated over a period of 8 minutes, n = 6; Fig. 8B). While MeAIB completely occluded retrograde signaling in n = 5 pairs, the first 13 conditioning trains (90 s) still produced IPSP depression in n = 1 in the presence of MeAIB (Fig. 8B”,B”’). However, the extent of this initial IPSP depression was progressively reduced as compared with those recorded in

![Figure 8. SAT2 is required to sustain retrograde glutamate release from pyramidal cell dendrites. (A) Vesicular glutamate release [arrow] from dendrites of layer 2/3 pyramidal cells has been proposed as a means to control short-term synaptic plasticity at inhibitory terminals of FS cells (FS) innervating pyramidal cells (Pyr). Inhibitory perisomatic terminal is depicted by solid black triangle. Current-clamp traces represent firing patterns of recorded neurons in response to somatic current injection. (B) Change in synaptic gain in the presence of K⁺ alone (n = 3), or upon exposure to K⁺ and MeAIB (n = 6) in control and after inducing action potential-based retrograde signaling by 10 bAPs (at 50 Hz) in the postsynaptic pyramidal cell 250 ms before synaptic activation by somatic stimulation of the FS cell ("conditioning"). Data were expressed as binned values over 90-s periods. Note the lack of IPSP depression upon MeAIB application throughout, pointing to a loss of glutamate available for retrograde release from pyramidal cell dendrites. Data represent means ± SEM. *P < 0.05; **P < 0.01 indicate significant IPSP suppression as compared with the averaged baseline value representing 1 (horizontal dashed line; Student’s t-test). (B’-B”’) IPSP distribution from representative dual whole-cell current-clamp recordings under control conditions (20 mM K⁺) and after sustained MeAIB application. MeAIB either partially (n = 1; B”) or completely (n = 5; B”’) inhibited retrograde glutamate signaling thus occluding IPSP depression. (C) Proposed molecular hierarchy of retrograde signaling by glutamate.](Cerebral Cortex May 2009, V 19 N 5 1101)
the absence of MeAIB (Fig. 8B’). Subsequent sweeps showed the lack of synaptic depression (Fig. 8B,B’) suggesting that residual glutamate may be present in pyramidal cell dendrites even upon extended preincubation with 20 mM K+ and MeAIB. The lack of metabolic substrate for de novo glutamate synthesis (Fig. 7C) upon synaptic activation led to the virtual elimination of retrograde signaling. Overall, these data demonstrate that SAT2-mediated glutamine transport is required for retrograde glutamate release to operate at subsynaptic dendrites of layer 2/3 pyramidal cells receiving synaptic input from FS cells, and define a novel metabolic function for SAT2 in the cerebral cortex.

**Discussion**

The results presented here demonstrate that SAT2 is preferentially localized in glutamatergic neurons in vitro and in vivo. Although SAT2 immunostaining is evident in mossy fibers, the most prominent staining is generally seen in the somatodendritic compartment of populations of glutamatergic neurons including neocortical pyramidal cells and principal cells of the hippocampus. In electrically stimulated nerve terminals and their corresponding subsynaptic dendrites the level of glutamate was sustained at the expense of glutamine, attesting to the role of glutamine in supporting glutamatergic signaling in vivo. As MeAIB blockage of system A transport consistently decreased the intracellular glutamate concentration and affected retrograde signaling controlling short-term plasticity at inhibitory synapses between FS cells and pyramidal cells in layer 2/3 of the neocortex, the capacity for glutamate release from primary cortical neurons appears substantially dependent on the bioavailability of extracellular glutamine. This observation suggests a role for glutamate in retrograde signaling and points to the involvement of SAT2 in the glutamate-glutamine cycle required for the continuous replenishment of dendritic glutamate pools.

**SAT2 Mediates Glutamine Uptake into Glutamatergic Neurons**

SAT2 was not detected in GABAergic cells in one study (Gonzalez-Gonzalez et al. 2005), while another report showed expression of SAT2 in all cortical GABAergic cells (Melone et al. 2006). The latter study also demonstrated SAT2 colocalizing with GFAP. Notably, the very same antibodies failed to produce any staining for SAT2 in cultured glial cells in other studies (Yao et al. 2000; Armano et al. 2002). SAT2 mRNA has been shown in glial cells by in situ hybridization (Reimer et al. 2000) and demonstrated by reverse transcription–PCR in cultured glial cells (Deitmer et al. 2003). However, SAT2-mediated glutamine uptake was not detectable in the latter astroglia cultures (Heckel et al. 2003). Our findings with highly specific affinity-purified antibodies resolve this controversy by demonstrating that the SAT2 isoform of the SATs is enriched in a subset of glutamatergic neurons in the CNS. Only weak, if any, SAT2 staining was seen in subsets of GABAergic interneurons, for example, in the hippocampus and neocortex. The lack of SAT2 expression in GABAergic neurons was supported by in vitro findings showing lack of SAT2 colocalization with GAD65. These findings were further corroborated by the marked differences in the regional distribution of SAT2: only weak immunolabeling was detected in the striatum, which contains cholinergic interneurons and GABAergic projection neurons, and in the pars reticulata and pars compacta of the substantia nigra that contain vast quantities of GABAergic and dopaminergic cells, respectively. In the hippocampus, we detected moderate staining of glial cells with one of the antibodies (SAT2-N2) we generated. In the rest of the brain, each of the 3 antibodies produced only faint staining of glia (including Bergmann fibers), indicating very low levels of SAT2 in these cells. In summary, our data demonstrate a selective localization of SAT2 in glutamatergic neurons, thus corroborating previous data on the enrichment of SAT2 mRNA in excitationary neurons (Reimer et al. 2000; Yao et al. 2000).

We have detected only low levels of immunoreactivity for SAT1, a SAT2 homolog, in glutamatergic neurons (unpublished data), in agreement with previous reports on the enrichment of mRNA for SAT1 in GABAergic cells (Chaudhry, Schmitz, et al. 2002). In addition, as none of the other major glutamine-recognizing transporters are expressed in glutamatergic neurons, including SN1 (Boulland et al. 2002), SN2 (Cubelos et al. 2005), and ASCT2 (Heckel et al. 2003), SAT2 is likely to be a major regulator of the glutamate-glutamine cycle in excitatory neurons. Indeed, SAT2 localization in the somatodendritic compartment complements in many brain regions the localization of SN1 which extrudes glutamine from glial cells (Boulland et al. 2002, 2003), thus supporting a SN1-SAT2-mediated glutamine shuttling between perisynaptic astroglia and neurons.

Energized by the coupled movement of Na+ and Ca2+, SAT2 generates steep concentration gradients of small aliphatic neutral amino acids (Reimer et al. 2000; Sugawara et al. 2000; Yao et al. 2000) with comparable affinities for alanine, proline and glutamine (Chaudhry, Schmitz, et al. 2002). However, in the cerebrospinal fluid, the glutamine concentration (~0.5 mM) by far exceeds that of any other amino acid that can enter through SAT2, such as alanine (<50 μM) (Hamberger et al. 1983). Under physiological conditions, glutamine will therefore compete out for example alanine for transport by SAT2. Thus, SAT2 may have an intrinsic role in the glutamate-glutamine cycle in the CNS. SAT2 may, inter alia, be responsible for the reported MeAIB-induced inhibition of hippocampal epileptiform activity, which requires sustained glutamate release (Bacci et al. 2002).

**Conversion of Glutamine to Glutamate during Synaptic Activity**

We have demonstrated activity-dependent release of glutamate from cultured cortical neurons. These data are in line with the reported Ca2+-dependent release of glutamate from cultured neurons (Oltedal et al. 2007) and hippocampal slices in vitro (Hamberger et al. 1979). We have also established that the level of released glutamate from cultured cortical neurons is dependent on the available extracellular glutamine. These data are in accordance with previous findings that adding glutamine in the depolarizing incubation buffer prevents the depletion of glutamate from nerve terminals (Storm-Mathisen et al. 1986; Gunderesen et al. 1998b), and also increases the release of glutamate (Szerb and O’Regan 1985). Moreover, application of exogenous glutamine stimulates the formation of glutamate and increases neuronal activity (Szerb and O’Regan 1984; Verderio et al. 1999). Indeed, radiolabeled glutamine preferentially labels the releasable pool of glutamate (Ward et al. 1983), and inhibition of glutamine synthesis by l-methionine sulfoximine leads to a partial depletion of...
presynaptic glutamate (Storm-Mathisen et al. 1986; Pow and Robinson 1994; Laake et al. 1995). Glucose alone is insufficient to maintain the generation of neurotransmitter and is unable to sustain glutamatergic transmission (Hamberger et al. 1979; Garcia-Espinosa et al. 2004; Hertz and Zielke 2004). This lends further support to neuronal glutamine uptake for synaptic function.

A recent study has shown that glutamate entering through system A activity increases hippocampal miniature excitatory postsynaptic current (EPSC) amplitude, but only after prolonged incubation (>4 h) (Kam and Nicoll 2007). Moreover, the authors showed that glutamate increases synaptic transmission in an activity-dependent manner, but that tissue slices can sustain synaptic transmission upon interruption of the glutamate-glutamine cycle. Although we observed SAT2 immunoreactivity in mossy fibers along their course in the stratum lucidum, we were unable to identify SAT2-stained glutamatergic terminals in the intact rat corticocolimnic system, including mossy fiber boutons. However, we demonstrate generation of glutamate at the expense of glutamine in the nerve endings of electrically activated perforant path. The lack of staining of the boutons could be due to a level of SAT2 below detection levels or it might be that SAT2 targeting to nerve terminals is regulated by synaptic activity, as has been shown for Kv4.2 potassium channels (Shibasaki et al. 2004). Alternatively, flow of glutamine from the cell body might replenish glutamine in the terminals. Our results showing pronounced expression of SAT2 and large amounts of glutamine in somatodendritic compartments can thus explain the demonstrated effect of MeAIB-sensitive glutamine uptake after prolonged incubations (Kam and Nicoll 2007) which could be explained by dendrites supplying nerve terminals with glutamine. However, terminals may also contain a yet unidentified glutamine transporter (Tamarappoo et al. 1997).

SAT2-Mediated Glutamine Transport in Retrograde Signaling

Our data demonstrate SAT2 expression in dendrites previously reported to release glutamate, for example, the dendrites of UBCs in the cerebellum and the pyramidal cells in the hippocampus and the neocortex (Mugnaini et al. 1997; Morishita and Alger 1999; Zilberter 2000; Harkany et al. 2004). Similarly, in the olfactory bulb, glutamate release has been demonstrated from the dendrites of mitral cells (Didier et al. 2001), and we find high levels of SAT2 in these dendrites.

Upon perforant path stimulation, the most severe reduction in glutamine concentration was seen in mitochondria of hippocampal granule cells. This appeared to be restricted to synaptically activated dendrite segments accumulating SAT2 in the MML, which (along with the outer zone of the molecular layer) receives input from perforant path axons. Mitochondria in the more proximal parts of the same dendrites, which are innervated by terminals of unstimulated mossy cells in the hilus, did not exhibit a similar reduction in their glutamine concentrations. Dendritic mitochondria distribution is regulated by synaptic activity and is associated with synapse plasticity (Li et al. 2004). Evidence has been presented that mitochondrial PAG, the key enzyme catalyzing the formation of glutamate from glutamine, is stimulated by Ca$^{2+}$ influx and by elimination of the negative feedback from its end product, glutamate (Kvamme et al. 1991; Roberg et al. 1995). Thus, depolarization of the dendrites with ensuing Ca$^{2+}$ influx and glutamate release may stimulate the accumulation of mitochondria at active sites and stimulate PAG activity, leading to enhanced consumption of glutamine for the formation of the neurotransmitter glutamate. Indeed, glutamine-derived neurotransmitter glutamate is not mixed with the endogenous “metabolic pool” of glutamate inside the mitochondria (Roberg et al. 1995).

Retrograde signaling provides essential feedback to control synaptic plasticity. Depolarization-induced suppression of inhibition depends on postsynaptic Ca$^{2+}$ entry and the formation and dendritic release of retrograde messengers. The spatial selectivity of retrograde signaling requires recruitment of unique presynaptic receptors and signal transduction machineries that control neurotransmitter release (Bliss and Collingridge 1993; Bolshakov and Siegelbaum 1994; Schuman and Madison 1994). Compelling evidence exists for glutamate being an endogenous retrograde messenger at several synapses (Alger and Pitzer 1995; Giltsch et al. 1996; Morishita and Alger 1999; Zilberter 2000). Accordingly, VGLUT3 is targeted to dendrites in a subset of neurons, including pyramidal cells in layer 2/3 of the neocortex (Fremeau et al. 2002). VGLUT3-mediated endocannabinoid-independent retrograde signaling has been shown at these dendrites (Harkany et al. 2004). Our present elucidation of dendritic glutamate release requiring SAT2-mediated glutamine uptake further supports a role for glutamate as a dendritic neurotransmitter as well as for SAT2 being an essential metabolic integrator of glutamate-glutamine cycling to sustain synaptic plasticity.

Electrogenic SAT2 transport depolarizes cells (Chaudhry, Schmitz, et al. 2002). Subthreshold depolarization may passively propagate from granule cell dendrites to mossy fiber boutons and enhance action potential-evoked transmitter release (Alle and Geiger 2006; Shu et al. 2006). Consequently, SAT2 in the dendrites of many excitatory neurons may also contribute to enhanced transmission at glutamatergic nerve terminals.

Conclusions

In summary, we have demonstrated that in the CNS, SAT2 has a cell-specific localization and is preferentially targeted to the somatodendritic compartment of glutamatergic neurons. We also show that these subcellular segments accumulate significant levels of glutamine which upon activation is metabolized in an “on-demand,” activity-dependent fashion, while intracellular levels of glutamate are sustained. Further, pharmacological disruption of SAT2 by MeAIB depletes intracellular glutamate and reduces glutamate mediated retrograde signaling from pyramidal cell dendrites to inhibitory nerve terminals in the cortical layer 2/3. Indeed, SAT2 localizes to dendrites previously shown to mediate retrograde signaling, such as dendrites of mitral cells, UBCs and hippocampal and neocortical pyramidal cells. We also demonstrate that glutamine serves as a precursor for glutamate generation in nerve terminals and we suggest the possible existence of yet unidentified transporters as well as a potential for SAT2 to be upregulated under certain conditions at nerve terminal membranes.

Supplementary Material

Supplementary material can be found at: http://www.cercor. oxfordjournals.org/
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