GLUTAMATE TRANSPORTERS AROUND THE TRIPARTITE SYNAPSE

by

Silvia Holmseth

Centre for Molecular Biology and Neuroscience

Department of Anatomy, Institute of Basic Medical Sciences,

University of Oslo

2011

Series of dissertations submitted to the
Faculty of Medicine, University of Oslo
No. 1239

ISBN 978-82-8264-287-3

Cover: Inger Sandved Anfinsen.
Printed in Norway: AIT Oslo AS.

Produced in co-operation with Unipub.
The thesis is produced by Unipub merely in connection with the
thesis defence. Kindly direct all inquiries regarding the thesis to the copyright
holder or the unit which grants the doctorate.

Table of contents

Acknowledgements……5

Abbreviations……6

Minireview ……7

Aims ……..20

Methods……21

Synopsis……28

- Summary……28

- Original papers……..29

- Comments on individual papers……...30

Conclusions…….. 38

References……40

Papers……53

Acknowledgements

The present work was performed at the Department of Anatomy, Institute of Basic Medical Sciences, University of Oslo and the Centre for Molecular Biology and Neuroscience (CMBN), Norway. I am grateful for the great facilities being provided to me.

I would like to thank my principal supervisor Niels Christian Danbolt and my co-supervisor Knut Petter Lehre for excellent guidance and for always taking their time. You have been great motivators and mentors ever since I was introduced to science when starting my master in 2001.

I am grateful to all my colleagues and collaborators who contributed to the papers in this thesis. Thanks to past and present co-workers in the Neurotransporter Group. Also special thanks for technical assistance to Henriette Danbolt. Also thanks to the directors of the department of Anatomy during these years. Finally, I would like to thank my closest family and friends for backing med up and believing in me.

The work has been funded by The Norwegian Advanced Research Program (Toppforskningsprogrammet).

Silvia Holmseth
Oslo, September 2011

Abbreviations

ALS	Amyotrophic lateral sclerosis
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
ASCT	Neutral amino acid exchanger
CNS	Central nervous system
DHK	Dihydrokainate
EAAT	Excitatory amino acid transporter (= glutamate transporter)
EAAC1	Excitatory amino acid carrier (EAAT3)
GAD	Glutamic acid decarboxylase
GDH	Glutamate dehydrogenase
GLAST	Glutamate aspartate transporter (EAAT1)
GLT1	Glutamate transporter (EAAT2)
GSH	Glutathione
GTRAP	Glutamate transporter associated protein
mGLUR	Metabotropic glutamate receptors
MSO	Methionine sulfoximine
NAC	Sodium dicarboxylate transporter
NMDA	N-methyl-D-aspartate
PAG	Phosphate activated glutaminase
PSD	Postsynaptic density
SLC	Superfamily of solute carriers
TBOA	*threo*-β-benzyloxyaspartate
VGLUT	Vesicular glutamate transporter
xCT	Glutamate-cystine exchanger

Minireview

In addition to being an amino acid and a component of proteins, glutamate is the main excitatory neurotransmitter in the central nervous system (CNS) (for review see: Fonnum, 1984; Ottersen and Storm-Mathisen, 1984; Danbolt, 2001). Glutamate is involved in most aspects of normal brain function including cognition, memory and learning. Brain tissue contains large amounts of glutamate, around 5-15 mmol per kg depending on the region (Schousboe, 1981). The extracellular concentrations are kept low and are in the order of a few micromolar (Hamberger et al., 1983), or may be even lower (Herman and Jahr, 2007). The highest glutamate concentrations are found intracellularly in glia cells, nerve terminals and synaptic vesicles (in increasing order) (Ottersen et al., 1992). It is suggested a concentration of more than 60 mM inside synaptic vesicles (Shupliakov et al., 1992). The concentration in cytosol is not known, but assumed to be in the low millimolar range implying that the concentration gradient across the plasma membrane is several thousand fold.

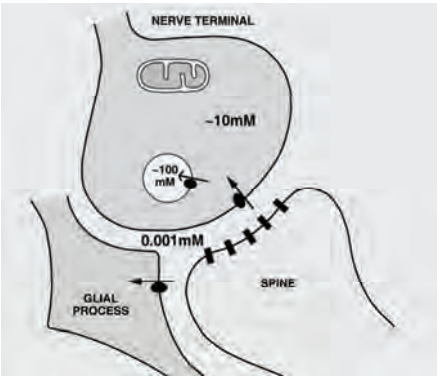


Figure 1: The concentration gradients across the plasma membranes are great. The extracellular glutamate concentration is around 1 μM, inside nerve terminals it is around 10 mM and 100 mM in synaptic vesicles (for references see: Danbolt, 2001*).*

When glutamate has been released into the extracellular space, it binds to glutamate receptors on neuronal and glial cell membranes to exert its signaling role. The glutamate receptors are divided into three families (for review see: Kristensen et al., 2006). One

family is the metabotropic receptors which are coupled to G-proteins (mGluR1-8). Activation of these receptors leads to changes in inositol phosphate or cyclic nucleotide metabolism. The two other families are ionotropic receptors which mean that they are glutamate gated ion channels that conduct Na^{+} or Ca^{2+}. They are named after the glutamate analogues that activate them. One of them is NMDA (N-methyl-D-aspartate) receptors which has high affinity for glutamate and are slowly inactivating. The last family is AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) or kainate receptors according to their preference for AMPA or kainate. AMPA/kainate receptors are rapidly inactivating and have low affinity for glutamate.

Because glutamate modulates fundamental neurological processes and because glutamate only can act on the glutamate receptors from the outside, the extracellular concentrations must be tightly controlled. Both too much and too little receptor stimulation can be harmful l(Danbolt, 2001). There is no enzyme extracellularly that can metabolize glutamate, so the glutamate must be removed by cellular uptake (for review see: Danbolt, 2001). Although simple diffusion may be important for the reduction of glutamate in the synaptic cleft in the submillisecond timescale (Clements, 1996), diffusion can only cause glutamate redistribution. It cannot cause real removal from the extracellular fluid. The only mechanism for net removal is cellular uptake mediated by the glutamate transporters located in both neurons and astroglia (for review see: Danbolt, 2001).

The close proximity of astrocytes to synapses points towards glia as a part of the synaptic functional unit. The term “the tripartite synapse” has been introduced in recognition of the contribution of astrocytes to synaptic function (Volterra et al., 2002). In addition to clearance of neurotransmitters, it has been proposed that astrocytes can have neuron–like activities like releasing glutamate (Bezzi and Volterra, 2001). This, however, is still debated. For instance, a recent transcriptome database does not give support to the notion that astrocytes express the proteins involved in vesicular glutamate release (Cahoy et al., 2008) and it remains to be shown if glutamate containing vesicles exists in astrocytes.

Glutamate transporters

Glutamate transporters are also called "sodium and potassium coupled glutamate transporters" or "excitatory amino acid transporters" ("EAATs"). The term "sodium dependent high-affinity transporters" have earlier been in use, but because the affinity not is particular high (K_m varying between 1-100 μM depending on the subtype and assay method) and because the transporters also depend on potassium, this term is no longer in common use (Danbolt, 2001). Five distinct glutamate transporter subtypes have been cloned from animal and human tissue and have been assigned to the solute carrier family (slc) number 1: GLAST (EAAT1; slc1a3; Storck et al., 1992), GLT1 (EAAT2; slc1a2; Pines et al., 1992), EAAC1 (EAAT3; slc1a1; Kanai and Hediger, 1992), EAAT4 (slc1a6; Fairman et al., 1995), and EAAT5 (slc1a7; Arriza et al., 1997). In addition several splice-variants of the various EAATs have been reported (Utsunomiya-Tate et al., 1997; Meyer et al., 1998a; Münch et al., 1998; Meyer et al., 1999; Huggett et al., 2000; Rauen et al., 2004; Rozyczka and Engele, 2005). Two transporters for neutral amino acids (alanine serine cysteine transporter; ASCT1 and 2; gene slc1a4 and 5) have also been assigned to this family. There are also other transporters capable of transporting glutamate. For instance, in mitochondria there are "mitochondrial glutamate transporters" (for review see: Sluse, 1996) and in synaptic vesicles (Varoqui et al., 2002; Chaudhry et al., 2008) there are "vesicular glutamate transporters" (VGLUT1-3; slc17a6-8). In the plasma membrane there are transporters for neutral amino acids (e.g. ASCT2; slc1a5) and dicarboxylates (e.g.NAC3; gene slc13a5) that can transport glutamate with low affinity. In addition there is a glutamate-cystine exchanger (xCT; slc7a11) that exchange extracellular cystine with intracellular glutamate (Sontheimer, 2003; Sato et al., 2005).

Transport mechanism and stoichiometry

Glutamate transporters use the electrochemical gradients of Na^+, K^+ and H^+ to transport glutamate into the cells (Kanner and Sharon, 1978). In that way they are secondary active transporters because the gradients for Na^+ and K^+ are maintained by the Na^+/ K^+-ATPase which requires ATP to work. The transport is electrogenic because more positive charge moves in than out in each transport cycle. The transport is therefore stimulated by the negative membrane potential. The glutamate transporters can work in both directions and

are described as shuttles that transport either glutamate, Na^+ and H^+ or K^+. When glutamate/ Na^+/ H^+ is transported into the cell, either K^+ or glutamate/ Na^+/ H^+ have to be transported out before starting a new transport cycle. Na^+ is required for glutamate binding and K^+ is required for net transport. In the absence of K^+, the transporters are locked in exchange mode and can only exchange external substrate with internal substrate (Danbolt, 2001). It is important to note that failed cycles occur in between completed (net) transport cycles (see Volterra et al., 1996). Exchange can be considered incomplete or as failed transport cycles. The stochiometry (the fixed number of ions involved in the transport) of the transporters have been determined to be 1 glutamate, 3 Na^+, 1 H^+, 1 K^+ for EAAT1-3 (Zerangue and Kavanaugh, 1996a: Levy et al., 1998; Owe et al., 2006). This is an important parameter as it determines the concentrative capacity of the transporters, their energy consumption and the sensitivity to changes in ion gradients. In addition to the stoichometric transport the transporters also function as ion channels (in particular EAAT4 and 5, but almost non-existing in GLT1. For review see Seal and Amara, 1999) and as water channels (at least in the case of EAAT1) (MacAulay et al., 2001).

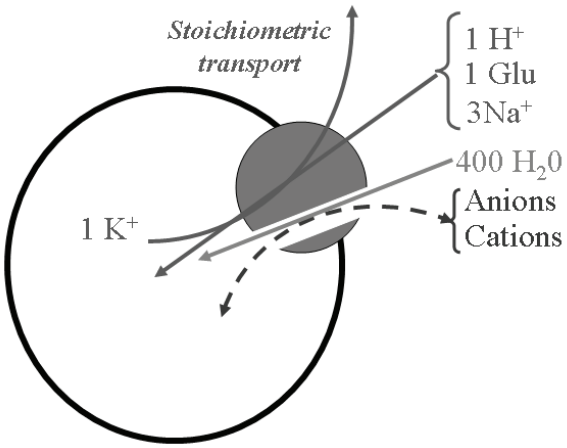


Figure 2: The stoichiometric transport of glutamate transporters. The transporters operate as shuttles that catalyze the stoichiometric transport of 1 glutamate, 3 sodium and 1 hydrogen ion

inwards in exchange with 1 potassium ion. In addition 400 water molecules are transported to the inside and the transporters can operate as ion channels.

Substrate selectivity

The glutamate transporter's specificity for glutamate is not absolute, because they can transport L-glutamate, D-aspartate, L-aspartate and L-cysteate with nearly the same affinity. In addition several analogs have been made. The glutamate molecule is very flexible and can have many different conformations. This explains how transporters, receptors and enzymes can bind glutamate despite having very different binding sites. This is what medicinal chemists exploit when they construct new molecules mimicking different glutamate conformations. Transportable glutamate analogs with high affinity include *threo*-β-hydroxyaspartate (THA), L-*trans*-pyrrolidine-2,4-dicarboxylic acid, L-serine-O-sulfate (L-SOS) and (2S,1'S,2'R)-2-(2-carboxycyclopropyl)glycine (CCG-III). There are also some non-transportable analogs, blockers, like dihydrokainic acid (DHK) and *threo*-β-benzyloxyaspartate (TBOA) derivatives (for review see: Bridges et al., 1999; Shimamoto and Shigeri, 2006).

Glutamate transporter structure

EAAT1-5 are glycoproteins consisting of between 500 and 600 amino acids. Their molecular masses are in the range of 60-75 kDa. They share 50-60 % amino acid sequence. Parts of the sequence are highly conserved between the EAATs, suggesting these parts play an important role. For instance, residues 396-400 (GLT1 nomenclature) are shown to be important for transport activity and to participate in sodium binding (Zarbiv et al., 1998; Zhang et al., 1998). And the residues 403-404 appear to be involved in potassium binding (Kavanaugh et al., 1997). Based on the crystal structure of a bacterial glutamate transporter homologue (Yernool et al., 2004), the transporters are predicted to have 8 transmembrane domains with intracellular amino- and carboxy-terminals. It is bowl-shaped with a large aqueous basin which makes glutamate easily reach binding sites halfway into the membrane, this architecture is well suited for rapid binding of glutamate in synapses. It has a triangular shape with sides of 80 Å. They most

likely both exist and work as trimers (Haugeto et al., 1996; Koch and Larsson, 2005; Grewer and Rauen, 2005).

Localizations and quantifications

The five different EAATs are found in different amounts in different areas of the brain. Quantification in absolute terms is required to know which glutamate transporter subtypes are dominating in different brain regions. The absolute amounts are also needed to determine their capacity in mathematical models (e.g. Rusakov and Kullmann, 1998) and to answer whether there is enough of the protein for its proposed role. Immunohistochemical studies have suggested that GLAST and GLT1 are both expressed in the plasma membranes of astrocytes facing neuropil (Lehre et al., 1995; Chaudhry et al., 1995; Lehre and Danbolt, 1998; Furness and Lehre, 1997; Furuta et al., 1997a; Furuta et al., 1997b; Furuta et al., 1997a Shibata et al., 1997). It is also shown that GLAST and GLT1 coexist in the same astrocytic membrane (Lehre et al., 1995; Haugeto et al., 1996), but as different homooloigomeric complexes (Haugeto et al., 1996). GLAST is the major glutamate transporter in the cerebellum (Lehre et al., 1995), the inner ear (Furness and Lehre, 1997; Takumi et al., 1997), the circumvenrtricular organs Berger and Hediger, 2000) and in the retina (Derouiche and Rauen, 1995), while GLT1 is the quantitative dominating glutamate transporter in the forebrain: about 1% of total brain protein is GLT1 (Danbolt et al., 1992; Lehre and Danbolt, 1998). GLAST and GLT1 are expressed at low levels at birth, but increases dramatically during development (Ullensvang et al., 1997). The highest increase is during synaptogenesis (P14-P28). The concentrations of GLAST and GLT1 are very high: 15 000 and 23 000 molecules per μm^3 in the stratum radiatum of hippocampus and molecular layer of cerebellum, respectively (see also Table 1 below). By employing a stereological method to estimate the cell membrane area containing these transporters, it was possible to calculate the density of transporters in the membranes (Lehre and Danbolt, 1998).

EAAT4 is mainly found in the Purkinje cell spines and dendrites in the cerebellar molecular layer (Dehnes et al., 1998). It is expressed at low levels in the forebrain. In the cerebellum the amount of EAAT4 is 0.2 mg/g tissue giving a density of 1800 molecules per μm^2 spine membrane.

EAAT5 is only found expressed in retina (Arriza et al., 1997), where it is localized in Müller cells and neurons (Eliasof et al., 1998). It seems that the associated chloride conductance is more important for EAAT5's physiological role than the transport function (Veruki et al., 2006; for review see Wadiche and von Gersdorff, 2006).

EAAC1 is a neuronal glutamate transporter (see paper IV included in this thesis).

GLT1 splice variants

The mRNA encoding GLT1 is large (11 kb; Pines et al., 1992) and has been found to have more than 30 splice variants; some of which translate into variant proteins (Utsunomiya-Tate et al., 1997; Meyer et al., 1998b; Münch et al., 1998; Meyer et al., 1999; Rozyczka and Engele, 2005; Rauen et al., 2004). Alternatively splicing of both N- and C-terminal exist. The functional properties are not changed by altering the termini (Sullivan et al., 2004). There exist at least three different C-terminal GLT1-variants (Rauen et al., 2004). The first studies on GLT1 protein localization (Danbolt et al., 1992; Lehre et al., 1993; Levy et al., 1993; Chaudhry et al., 1995; Lehre et al., 1995) used antibodies recognizing all isoforms. Subsequent antibodies (Rothstein et al., 1994; Schmitt et al., 1996) raised to the extreme C-terminus of GLT1a gave a seemingly identical labeling pattern. In contrast, the first reports on the distribution of GLT1b (protein and mRNA) in the brain were conflicting as some investigators detected it in neurons (Schmitt et al., 2002; Chen et al., 2002; Kugler and Schmitt, 2003; Reagan et al., 2004), including nerve terminals (Chen et al., 2002), while others only observed astroglial expression of the protein (Reye et al., 2002; Sullivan et al., 2004). Chen and co-workers later concluded that their anti-GLT1b antibodies were not specific (Chen et al., 2004). Instead they found that antibodies to GLT1a labeled (in hippocampus CA1) a subset of axon terminals and spines in addition to astroglia. Berger and co-workers (2005) demonstrate that the probes used for detection of GLT1b mRNA have an unspecific component, and conclude that both isoforms are widely expressed in astrocytes and that GLT1a is the predominant neuronal isoform. The expression of GLT1a protein in terminals was confirmed in the hippocampus (Furness et al., 2008) and in the somatic sensory cortex (Melone et al., 2009). Four different GLT1 N-terminal

variants also exist (Utsunomiya-Tate et al., 1997; Rozyczka and Engele, 2005), although it is unknown which of N-terminal variants that are expressed in the CNS in vivo.

Glutamate is recycled (the glutamate-glutamine cycle)

From the described localization and quantification data above it follows that the majority of glutamate uptake is catalyzed by GLT1 which is mainly localized in glia (Paper V). In order to be recycled, this means that the glutamate must be further transported to get back to the nerve terminals. In glia glutamate is detoxified to glutamine by glutamine synthetase. Glutamine is transported out of the glia cell by glutamine transporters and taken up by nerve terminals by another glutamine transporter. Inside mitochondria of the nerve terminal glutamine is reconverted to glutamate by the neuron-specific phosphate activated glutaminase (PAG). The glutamate is then loaded into vesicles by vesicular glutamate transporters (VGLUTs) and ready for use again.

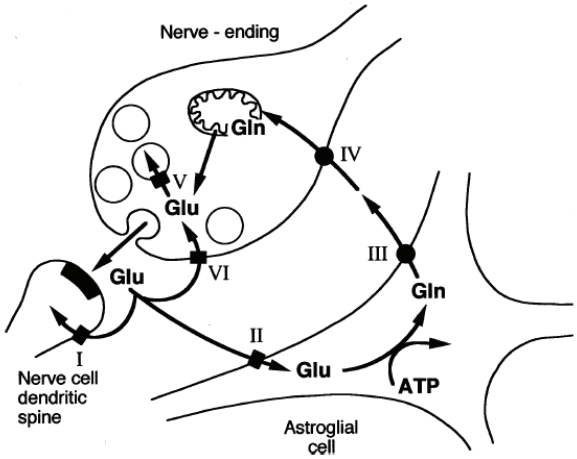


Figure 3: Recycling of glutamate by the glutamate-glutamine cycle. Glutamate is released from the nerve terminal and taken up by glutamate transporters in the dendritic spine (I), the nerve terminal (VI) and in the glial cell (II). Inside the glial cell it is detoxified to glutamine by glutamine synthetase, released to the extracellular space by a glutamine transporter (III) and taken up in the neuron by another glutamine transporter (IV). (Reproduced from Danbolt 2001).

The importance of the glutamate-glutamine-cycle is discussed, but there are several evidences for its importance. Inhibition of glutamine synthetase by methionine sulfoximine (MSO) leads to a loss of synaptic activity in the retina and depletion of neuronal glutamate (Laake et al., 1995; Barnett and Pow, 2000). Inhibition of glutamine synthetase also inhibits epileptiform activity on hippocampal cultures (Bacci et al., 2002). Also the majority of the glutamate transporters are localized in the proximity of synapses and glutamine synthetase is exclusively a glial enzyme while PAG is neuronal. On the other hand, de novo synthesis of glutamate from glucose has considerable function because each glutamate molecule, on the average, only can be recycled 3-4 times before being oxidatively degraded by the Krebs cycle (Hertz et al., 1999). Glutamatergic neurons can also sustain glutamate release independent of glutamine by pyruvate carboxylation (Hassel and Bråthe, 2000), although it has earlier been reported that pyruvate carboxylase is an astrocytic enzyme (Yu et al., 1983).

Uptake of glutamate into nerve terminals

It seems bothersome to do this recycling through glial cells. The most efficient would be direct uptake of glutamate into the nerve terminals. On the other hand, the uptake is electrogenic contributing to depolarization, and this may be a reason why evolution has favored sharing the burden with astrocytes. This uptake has been a mystery. Several studies have shown that this uptake exists, but the protein responsible has unvaded detection (see comments to Paper V).

Regulation

Glutamate uptake can be regulated on virtually all levels: DNA transcription, mRNA splicing, protein synthesis, targeting, glutamate transport and associated ion channel activity (Danbolt, 2001). A number of substances and proteins have shown to affect uptake activity and expression of glutamate transporters. Interestingly, several regulatory mechanisms can have differential effect on different subtypes of glutamate transporters.

Dependence of neurons for glial glutamate transporter expression is also shown in vivo when GLAST and GLT1 levels are reduced in striatum after lesioning of afferent

glutamatergic nerve fibers (Levy et al., 1995; Ginsberg et al., 1995). When astrocytes are cultured in the presence of neurons they express both GLT1 and GLAST, while pure astroglial cultures only express GLAST (Gegelashvili et al., 1997a; Swanson et al., 1997). Glutamate uptake is thought to be modulated by it's substrate glutamate. When glutamate binds to glutamate receptors, it is thought that this may represent a feedback regulatory mechanism for glutamate uptake. Long-term treatment of astrocytes in culture with L-glutamate (0.1-3 mM) resulted in a dose-dependent increase in uptake activity (Gegelashvili et al., 1996). In addition glutamate uptake is modulated by fatty acids like arachidonic acid (Trotti et al., 1995; Zerangue et al., 1995; Dunlop et al., 1999), by phosphorylation (for review see Gonzalez and Robinson, 2004), by red-ox mechanisms (Volterra et al., 1994; Trotti et al., 1997), and by growth factors, cytokines and soluble factors (Gegelashvili et al., 1996; Gegelashvili et al., 1997b; Schlag et al., 1998). Glutamate transporters are kept in position and modulated by proteins connected to the cytoskeleton. Four such proteins have so far been characterized: Ajuba which binds GLT1 (Marie et al., 2002), GTRAP3-18 which binds EAAC1 (Lin et al., 2001), and GTRAP41 and GTRAP48 that binds EAAT4 (Jackson et al., 2001). Increased GTRAP3-18 expression in cells reduces glutamate transport by lowering substrate affinity of EAAC1. This is also seen in another study where glutathione is decreased when GTRAP3-18 expression is increased, then GTRAP3-18 modulate EAAC1s uptake of cysteine which is necessary to make glutathione (Watabe et al., 2008). GTRAP3-18 might act as an endogenous inhibitor of EAAC1. On the other side Ajuba is not found to alter glutamate transport mediated by GLT1, Ajuba's function with respect to glutamate transport is unclear. NMDA-receptor subunits also interact with EAAC1 to control surface expression, suggesting a close relationship between glutamate receptors and transporters (Waxman et al., 2007). In general, the machinery for EAAC1-trafficking seems to be more developed than for the other transporters. Which is logical since EAAC1 is expressed in both kidneys and intestine (Paper IV) where metabolic needs rapidly changes. Further evidence of differential regulation of glutamate transporter subtype expression is seen during development (Ullensvang et al., 1997, Furuta et al., 1997a). Rothstein et al. (2005) reported that β-lactam antibiotics (e.g. ceftriaxone)

selectively elevate GLT1 expression. Ceftriaxone appeared to be cytoprotective both in vitro and in vivo in a mouse model of ALS (Rothstein et al., 2005).

The importance of glutamate transporters

As shown (Lehre and Danbolt, 1998) the concentration of the major glutamate transporters are very high, 15 000 and 23 000 molecules per μm^3 in the stratum radiatum of hippocampus and molecular layer of cerebellum, respectively. Whether the glutamate transporters manage to clear the synaptic released glutamate will depend on several factors. First, it depends on how much glutamate is released. One μm^3 contains about one glutamatergic nerve terminal (Woolley and McEwen, 1992). It is assumed that one vesicle is released at the time, and that each vesicle contain some 400-5000 glutamate molecules (Clements, 1996), which is a broad range. Second, the morphology of the synapse matters. Most of the glutamate transporters are located on astrocytes. If the transporters shall contribute to clear the glutamate, they must be located near the glutamate release sites. Because localization of astrocytes in relation to synapses varies between regions and different synapses, the role of glutamate transporters will also vary. Third, the number of glutamate transporters will be important. The high number of glutamate transporters are sufficiently high to clear the number of glutamate molecules released per vesicle. Since the glutamate transporters are slow (about 70 ms per cycle (Wadiche et al., 1995; Auger and Attwell, 2000), they compensate by their high number and high affinity that easily make them "buffer" the extracellular glutamate. GLT1 clears most of the glutamate with its high number and localization in astrocytic membranes close to synapses, GLAST and EAAC1 seem to play minor roles. It is likely that glutamate transporters serve at least dual functions both as a transporter of other substances than glutamate and as an ion-channel (Ryan et al., 2004; Veruki et al., 2006). Why there are five different glutamate transporters and several splice variants that are so differentially expressed and regulated is a question.

The physiological roles of glutamate transporters

It is obvious that glutamate transporters play a significant role in removing glutamate from the extracellular space. The abundance and importance of GLT1 is also evident from the phenotype of GLT1 deficient mice. In mice deficient in GLT1 glutamate levels

rise enough to cause epilepsy and cell death (Tanaka, 1997). Similarly, the GLAST knockout mouse show reduced motor coordination and have major changes in the retina (Watase et al., 1998), which is in agreement with the localization of GLAST. The EAAC1 knockout do not develop remarkable neurological symptoms, except for reduced spontaneous locomotor activity and dicarboxylic aminoaciduria (Peghini et al., 1997). EAAC1 also transports cysteine with an affinity 10–20-fold higher than that of GLAST or GLT-1 (Zerangue and Kavanaugh, 1996b). A recent study demonstrated age-dependent neurodegeneration with decreased glutathione (GSH) content, increased oxidant levels and increased susceptibility to oxidative stress in EAAC1-deficient mice (Aoyama et al., 2006). These EAAC1-deficient mice also showed an age-dependent decrease in neuronal number in the substantia nigra (Aoyama et al., 2008a). GSH plays an important role in detoxifying reactive oxygen species (ROS) and thereby protecting cells from oxidative stress (for review see Dringen, 2000). GSH is a tripeptide composed by cysteine, glycine and glutamate where cysteine is the rate-limiting factor for GSH-synthesis in neurons. In primary neuron culture, approximately 90% of total cysteine uptake is mediated by sodium-dependent systems, mainly excitatory amino acid transporters (EAATs) (Chen and Swanson, 2003; Himi et al., 2003).

Glutamate transporters and disease

Malfunction of glutamate uptake has been reported in many diseases, but it is not easy to differentiate between cause and effect of dysfunction of glutamate transporters (for review, see Danbolt, 2001;Beart and O'Shea, 2007). The role of glutamate transporters in disease are often connected to glutamate as a neurotoxin. At high extracellular concentrations glutamate is toxic to the brain and can contribute to neuronal cell death (Choi, 1992). If the extracellular glutamate is increased more glutamate will bind to glutamate receptors. Binding to the ionotropic glutamate receptors will give more glutamate release and an influx of Na^+ and Ca^{2+}. The cells energy consumption will increase in order to pump these ions out again. In addition free radical production will increase (Bondy and Lee, 1993). This may in turn impair energy production and glutamate uptake, and maybe even reverse glutamate transporters causing further

glutamate release. An increase in extracellular glutamate concentration easily starts vicious circles.

In ischemia, hypoxia and hypoglycemia, ion gradients dissipate, glutamate transporters reverse and a massive efflux of glutamate and cell death is the result (Nicholls and Attwell, 1990; Rossi et al., 2000). In addition there is a vesicular release (Drejer et al., 1985) and release through swelling-activated anion channels (Kimelberg and Mongin, 1998). It is still debated from which compartments most of the glutamate leak during ischemia (Ottersen et al., 1996; Lipton, 1999). The most favored hypothesis is that the glutamate comes from neurons because most brain glutamate is stored there. Further, the glutamate uptake in glia cells are less sensitive to hypoxia than the neuronal uptake (Swanson et al., 1994). Quantitative immunocytochemistry studying the changes in glutamate concentrations following brain ischemia strongly suggest that the release of glutamate is by neurons (Ottersen et al., 1996), and by the dendro-somatic compartments.

ALS-amyotrophic lateral sclerosis is characterized by muscular weakness, atrophy and spacticity (Chancellor and Warlow, 1992). It is caused by death of motor neurons where exitotoxicity is one possible reason. A dramatic loss of GLT1 in ALS-patients have been shown (Rothstein et al., 1995), but another paper has shown no change (Milton et al., 1997). Whether reduced GLT1-expression is a primary event leading to motor neuron death or if it is secondary to the neuron death is not clear.

Also in Alzheimer's disease there are several contradictory findings about reduced levels of GLT1 (Li et al., 1997) while another report found no correlation between reduced GLT1 levels and Alzheimer (Beckstrøm et al., 1999). It has been suggested that oxidation is a cause of disturbed GLT1 expression and that GLT1 splice variants occur in several neurological diseases like Alzheimer and ALS (Honig et al., 2000).

It has been observed elevated levels of glutamate in patients with various epilepsy (e.g. Janjua, 1992). And mice deficient of GLT1 develops spontaneous seizures (Tanaka, 1997). In contrast to the mentioned neurological disorders it is questioned if it is too much glutamate transport in schizophrenia (for review, see: Carlsson et al., 1997). A glutamatergic hypofunction can be due to hyperactive glutamate transporters, defective receptors, lack of receptors, inadequate glutamate release or lack of glutamatergic nerve terminals.

AIMS

The overall aim of this thesis has been to increase our insight into glutamatergic transmission and reuptake by providing quantitative data on the distribution and expression levels of the various glutamate transporter subtypes. The thesis has specially searched to give answer to these questions:

-What is the functional significance of EAAC1?

-Which transporter is responsible for the uptake of glutamate into nerve terminals?

- How abundant are the various C-terminal variants of GLT1?

Methods

I will here give an overview of the main methods I have used in this study focusing on principles without repeating all the details as they are described in the papers.

Tissue preservation-fixation

The purpose of fixation is to preserve the tissue components of interest after death and during the cutting and labeling procedures. It is important to realize the type of fixative and the type of fixation procedure have to be adapted to the purpose. The fixation should preserve the structures without impairing detection. Because fixation is usually only the beginning of a long process, and it is important to consider the entire procedure before fixing the tissue.

Fixatives: These are chemicals that chemically modify the tissue in such way that the desired preservation is obtained. The most common is formaldehyde which binds to and make covalent bonds between adjacent amino groups. Formaldehyde has a tendency to polymerize, and the polymer diffuses slower into the tissue. To get an efficient and quick fixation it is important to work with monomers. This is usually accomplished by depolymerizing paraformaldehyde to formaldehyde shortly before use. Glutaraldehyde works in the same way, but is a slightly larger molecule which diffuse slower, but being a di-aldehyde it forms cross-links more efficiently. This is an advantage with respect to immobilizing diffusible molecules and with respect to preservation of ultrastructure, but not when it comes to antigen accessibility. Note that fixation time, concentration and temperature are just as important parameters as the choice of chemical compounds.

Fixation procedure: Small tissue pieces can be efficiently fixed simply by immersing them in fixative. The best fixation of thicker tissue (like a whole brain) is obtained by a perfusion of the animal through the heart. The animal is given a lethal dose of pentobarbital, then a tubing with a needle is put into the left ventricle-aorta and the fixative delivered through the tubing by a peristaltic pump.

Preparation of antibodies

Antibodies are immunoglobulins, proteins which specifically recognize their antigens. They are composed of two heavy and two light chains and are Y-shaped, the two heavy

chains are connected together by disulfide-groups. Between the light and the heavy chain there is an antigen binding site. In research antibodies are used to identify and localize proteins.

Antibodies have been made by immunizing animals with the antigen of interest. We have mostly used synthetic peptides corresponding to parts of the protein. The advantages with using short peptides are that we can avoid conserved parts and thereby avoid cross-reaction with other proteins. Further we know exactly which part of the protein it reacts to and the antibody can so be used in functional studies of the transporter proteins. The disadvantage with antibodies against short peptides is that they may not recognize the native protein, only the peptide, and it can be difficult to choose the most antigenic parts of the protein. Special computer programs exist that can help us choose the most hydrophilic and assumed most antigenic parts of the protein. But the programs only give an indication.

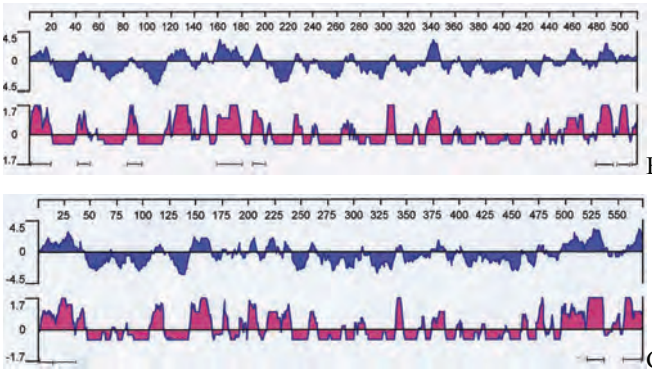


Figure 4: Antigenicity plot of EAAC1 and GLT1. Hydrophilic parts are shown in blue and the predicted antigen parts in pink.

It can be good to avoid transmembrane parts, as well as glycosylated and lipidated parts. Also cysteines should be avoided as they tend to make trouble during synthesis (Danbolt et al., 1998). But there are no absolute rules. From Figure 4 one can think that it should not be more difficult to produce antibodies to EAAC1 than to GLT1, but our experience and paper I shows that this is not the case.

The peptides must be coupled to a carrier as they are too small to be antigenic (molecules less than 1-5 kDa are normally not antigenic). We have used keyhole limpet hemocyanin (KLH), BSA or thyroglobulin as carrier proteins. The peptides are then coupled to the carrier by glutaraldehyde. Most often we have used rabbits for the production. They respond well and give 70-80 ml serum per month. The antibodies are affinity purified by immobilized peptide, the same as the animal was immunized with. If the antibody show unwanted reactivity, they should also be absorbed. Then the carrier-protein is coupled to agarose and aldehyde treated, and the sera is run slowly through this column.

Immunohistochemistry

Immunohistochemistry followed by microscopy is useful for studying a protein's localization at regional, cellular and subcellular level. Multiple labeling can also be done with different fluorochromes in order to compare labeling of several proteins at the time and see whether they colocalize or not.

Briefly, mice or rats are perfusion fixed, tissue taken out and cut on vibratome. The sections are blocked and incubated with primary antibody. The immunoreagents will diffuse into the section from the surface. The antigen-antibody complexes are visualized with biotinylated anti-rabbit IgG and streptavidin-biotinylated horseradish peroxidase complex followed by diaminobenzidine (DAB) for ordinary light microscopy or fluorescent dye for confocal microscopy.

Pre-embedding peroxidase labeling: Pre-embedding means labeling of the tissue before it eventually is embedded and processed for electron microscopy. Pre-embedding peroxidase labeling transforms the substrate diaminobenzidine into a precipitate that will be restricted by the cell membranes. This precipitate is electron dense so that the sections can also be used for electron microscopy if wanted. This is a sensitive method and has the advantage that the sections can be viewed light microscopically first. Interesting parts of the section can be cut out and embedded in a plastic material (Durcupan®) for electron microscopy. The method is not quantitative as structures may be unlabelled either because they do not contain the antigen or because the antigen is not accessible.

Post-embedding immunogold: Post-embedding labeling means that labeling is done after embedding and cutting of the sections. The antibody-antigen complex is viewed with a secondary antibody coupled to a gold particle (the gold particle is electron dense) This method is quantitative and gives higher resolution as it can show in which parts of the membrane the protein is localized, but it is less sensitive than the pre-embedding labeling.

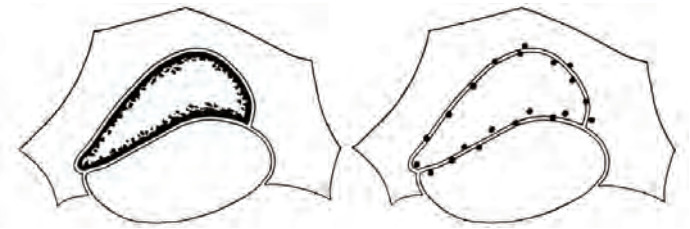


Figure 5. Illustration of pre-embedding (left) and post-embedding (right) techniques. For pre-embedding the reaction product is restricted by the cell membrane. For post-embedding the immunoreagents are not restricted by cell membranes. The length of the antigen-antibody complex is around 20 nm, so the gold particle can be on the outside of the structure although the antigen is on the inside (reproduced from Danbolt et al., 1998*).*

Microscopy

For ordinary light microscopy the resolution is limited by the wavelength of the light used. The resolution (d) is decided by the formula: $d=(0.61x\lambda)/NA$ where λ is the wavelength of the light and NA is the numerical aperture. Optimally the resolution can be 0.2 μm, but most often it is closer to 0.5 μm. This means that the light microscope is excellent for getting an overview over which part of the section and which structures that are labeled, but the subcellular labeling needs higher resolution. For instance nerve terminals which are about 500 nm will appear as dots in this microscope.

The electron microscope has high resolution and is extremely powerful for detecting the exact position of a protein at the ultrastructural level. Here the light is replaced by an electron beam, which has a wavelength of 0.005 nm. Since the resolution is proportional with λ it gives a very high resolution, normally around 0.2 nm

For fluorescent microscopy the antigen-antibody complex is viewed with a fluorescent dye (or particle like e.g. quantum dot) with a certain excitation and emission spectrum. Since fluorescent dyes exist with different emission spectra it can be performed multiple labeling. For multiple labeling you have to ensure that the emission spectra do not overlap. This allows us to study colocalization of proteins. The immunofluorescence technique requires application of antibodies which must be carefully validated by appropriate control experiments.

In a conventional (i.e., wide-field) microscope the entire specimen is evenly illuminated resulting excitation of the entire section thickness. Light originating from the parts of the section that are out of focus often causes a strong background. The background from unfocused parts can be avoided by using confocal microscopy. The confocal microscopy provides sharper images than the conventional microscope since it excludes light from outside the focal plane. Each image represents a thin cross-section of the specimen. It is also possible to obtain a three-dimensional reconstruction by sampling several images along the vertical axis of the specimen. The resolution of confocal microscopy is close to the theoretical limit (see above).

Western blotting

The purpose of blotting is to transfer the molecular species from the matrix of a gel (agarose or polyacrylamide) and to the surface of a membrane (nitrocellulose, PVDF, nylon or others) where they can be studied much more easily. In general proteins in a tissue homogenate are separated by molecular weight by SDS-PAGE (Laemmli, 1970). The proteins are subsequently transferred from a gel to a membrane The protein can then be immunolabeled with the desired antibody. Probed antigens can be visualized by a variety of secondary antibodies conjugated to e.g alkaline phosphatase or horseradish peroxidase. The immunocomplex can be detected by different methods like chemoluminecence, fluorescence or radioactivity. The method can be quantitative if specific controls or calibration systems are included. It is then important to work within the linearity of the signal. We used chemoluminecence which is a very sensitive method, the signal was captured by films or a CCD camera (a CCD camera converts optical

brightness into electrical amplitude using charge coupled device (CCD), the signals can be converted to digital values). Films are very sensitive, but the CCD camera can capture linear data over a broad range. The CCD camera gives digital recording and very short exposure times can be done. In such a way saturation during image acquisition can be avoided. It is very important to ensure precise loading of the gel and to repeat the experiments in triplicates.

Immunoblotting with purified protein extended the linear range upwards, but reduced linearity at low protein amounts presumably due to non-specific loss of protein. In paper IV this loss was prevented by adding 1 μg of total brain proteins from GLT1-deficient mice to all samples containing purified GLT1 protein (thus, the amount of purified proteins loaded was varied, but the amount of unrelated brain tissue proteins was kept constant). It is also important to save pictures from different exposure times in order to analyze the linearity of the exposure times.

Immunoisolation of proteins

Immunoisolation of proteins is purifying a protein by means of immobilized antibodies. The antibodies are first coupled to a solid phase material (like Protein-A-Sepharose Fast Flow, which is Protein A coupled to Sepharose beads). Protein A is the most known IgG binding protein. It is a Fc-receptor that means it can bind to the Fc part of the antibody, keeping the antigen binding site free to bind to the antigen. The antibody is then used to pull out the antigen from a tissue or cell extract. The antibody is covalently bound to the Protein-A-Sepharose by dimethyl suberimidate to ensure that the antibodies do not fall off in the last step when a low pH buffer is added. Brain tissue or transfected cells are homogenized in a suitable buffer. We have often used SDS as a detergent to be sure that the tissue is properly dissolved. After homogenization we have added Triton to make the solution antibody “friendly” before incubation with the immobilized antibodies. Immobilized antibodies were incubated with the extracts for 1–2 h before the proteins were eluted with a low pH-buffer The eluted proteins are then neutralized. Protease inhibitors and a reducing agent to avoid oligomerization of the transporter proteins are added to the eluate.

Cell culturing and transfection of mammalian cells

Cell culturing is a method where cells can be kept alive ex vivo. By keeping optimal conditions the cells can grow. By transfecting the cells with foreign DNA of a protein of interest, the cells can express the proteins we want. In paper IV we wanted to verify that the antibodies to the GLT1 subtypes also could distinguish between the individual splice variants on immunoblots (as brain tissue contains a mixture of them), HEK293T (human embryonic kidney cell cultures) cells were transiently transfected with cDNA encoding GLT1 variants, solubilized in SDS, subjected to SDSPAGE and immunoblotted with the antibodies. The HEK293T-cells did not have detectable endogenous expression of GLT1, neither non-transfected cells nor cells transfected with an open vector were immunopositive The GLT1 subtypes were immunoisolated with subtype specific antibodies from the transfected cells.

SYNOPSIS

Summary

Glutamate is the major excitatory neurotransmitter in the mammalian nervous system. It is inactivated by cellular uptake catalyzed by a family of glutamate transporter proteins (GLT1, GLAST, EAAC1, EAAT4 and EAAT5). The main aim of the present thesis was to determine the contributions of the individual glutamate transporter subtypes to the total glutamate uptake around hippocampal synapses focusing on the EAAC1 subtype and on the mysterious transporter responsible for nerve terminal uptake of glutamate. The first step on this endeavor was to make antibodies to EAAC1. As outlined in Paper I, it turned out to be more difficult to make good antibodies to EAAC1 than to the other glutamate transporters. Specificity testing using tissue from EAAC1 knockout mice as negative controls revealed highly specific interactions with unrelated proteins. Paper II summarizes of the lessons learnt about immunocytochemical specificity testing, and Paper III illustrates how the antigen pre-adsorption test can be misleading. After having overcome methodological problems, we were in position to address the original question. In Paper IV a new procedure for immunoisolation of EAAC1 was developed, and known amounts of pure EAAC1 protein was used as standard to quantify EAAC1 concentrations in brain tissue extracts. EAAC1 was found to be present at 13 μg per gram hippocampal protein. This is 100 times less than GLT1 and argues against a significant contribution of EAAC1 to rapid transmitter activation. EAAC1is selectively expressed in neuronal somata and dendrites throughout the brain, and thereby in a total surface area similar to that of astrocytes. In Paper V we show that nerve terminal glutamate uptake fully depends on GLT1, and that about 10% of hippocampal GLT1 protein is expressed terminals. This also explains why high levels of GLT1 mRNA is present in CA3 pyramidal cells. In Paper VI we describe antibodies to GLT1 splice variants and show that GLT1a represents about 90 % of total hippocampal GLT1, while GLT1b and GLT1c represented 6 and 1 %, respectively. We also provide the first data on the distribution of the GLT1b and show that this variant does not contribute to nerve terminal uptake.

Original papers included in this thesis:

I) Holmseth S, Dehnes Y, Bjørnsen LP, Boulland JL, Furness DN, Bergles D, Danbolt NC (2005) Specificity of antibodies: unexpected cross-reactivity of antibodies directed against the excitatory amino acid transporter 3 (EAAT3) Neuroscience 136: 649-660.

II) Holmseth S, Lehre KP, Danbolt NC (2006) Specificity controls for immunocytochemistry. Anat Embryol (Berl) 211: 257-266.

III) Holmseth S, Zhou Y, Follin-Arbelet V, Lehre KP, Bergles D, Danbolt NC
Specificity controls for immunocytochemistry: the antigen pre-adsorption test can lead to inaccurate assessment of antibody specificity
Submitted.

IV) Holmseth S, Dehnes Y, Huang YH, Follin-Arbelet V, Grutle NJ, Mylonakou NM, Plachez C, Bergles D, Zhou Y, Furness DN, Danbolt NC and Lehre KP, (2011)
Low density of EAAC1 (EAAT3; slc1a1) glutamate transporters suggests involvement in neuronal metabolism rather than in rapid control of synaptically released glutamate
Manuscript.

V) Furness D, Dehnes Y, Akhtar A, Rossi D, Hamann M, Grutle N, Gundersen V, **Holmseth S**, Lehre K, Ullensvang K, Wojewodzic M, Zhou Y, Attwell D, Danbolt N (2008) A quantitative assessment of glutamate uptake into hippocampal synaptic terminals and astrocytes: New insights into a neuronal role for excitatory amino acid transporter 2 (GLT1). Neuroscience 157:80-94.

VI) Holmseth S, Scott HA, Real K, Lehre KP and Danbolt NC (2009)

The concentrations and distributions of three C-terminal variants of the GLT1 (GLT1; slc1a2) glutamate transporter protein in rat brain tissue suggests differential regulation. Neuroscience 162: 1055-71.

Comments on original papers

Comments on paper I: Specificity of antibodies: Unexpected cross-reactivity of antibodies directed against the excitatory amino acid transporter 3 (EAAC1) glutamate transporter

Background: We wanted to quantify and localize EAAC1 in order to gain insight into its physiological functions. Because we initially did not have access to EAAC1 knockout mice, we made several antibodies to different parts of the EAAC1 protein molecule in order to be as sure as possible about the specificity. Our group had already made antibodies the C-terminus (residues 491-523 and 510-523) before I got involved. These antibodies gave rise to weak labeling on Western blots, but remarkably strong labeling in tissue sections. This mismatch made us feel uneasy. Another factor that made us feel uneasy was that preferential labeling of cytoplasm. Further, there were some data in the literature based on antisense knockdown of transporters attributing about a third of the total glutamate uptake activity to EAAC1 (Rothstein et al., 1996). We then noted that other investigators (Kugler and Schmitt, 1999) had made antibodies to a peptide corresponding to residues 480-499. They got strong labeling and described colocalization of EAAC1 and tubulin. We wondered if Rothstein and co-workers (1996) could be right after all.

Results: We made antibodies to a peptide similar to that of Kugler and Schmitt (1999), and like them, we also got antibodies that recognized a strong band. The electrophorectic mobility, however, was slightly higher than that of the band recognized by the other antibodies. Nevertheless, we got excited because this could mean that we were about to discover a novel and much more abundant variant of EAAC1. To be sure that this protein indeed was EAAC1, it was decided to try to immunoisolate it to get a protein sequence. Then we discovered that the protein was sometimes in the water soluble fraction and sometimes in the membrane fraction. This did not make sense. Further, the antibodies labeled axons strongly. Using a robotic ELISA system, I screened the antibodies for reactivity towards a number of non-EAAC1 proteins, including actin and tubulin which

are abundant in axons. The antibodies reacted strongly with tubulin and did so with a remarkable specificity. This explained why the protein was sometimes in the water soluble fraction: it was there when the conditions favored depolymerization of tubulin! I then fractionated the antiserum and obtained one fraction that recognized both tubulin and EAAC1, and another fraction that appeared specific for EAAC1. The EAAC1-specific fraction did not label axons. The labeling appeared restricted to the dendrosomatic compartment. There was no labeling of oligodendrocytes and no labeling of astroglia.

In parallel with this, we also made antibodies to a number of other EAAC1 peptides using different immunization protocols.

Discussion: This paper offers an explanation to why Kugler and coworkers observed a colocalization between EAAC1 and tubulin anf thereby concluded that EAAC1 is also in oligodendrocytes: their antibody probably also cross-reacted with tubulin. Unfortunately, their antibody was not available when we asked them for a sample so this hypothesis could not be tested directly. It is important to add that Kugler and co-workers only had one antibody and they did not have access to the EAAC1-knockout. This means that they did not have the means to uncover the reactivity with tubulin. Further, I had a robot to do the ELISA assays for me. During this work it became clear to us that the majority of antibodies against synthetic peptides do not recognize the native protein, and that cross-reaction with unrelated proteins is very common. Importantly, it also suggests that the pre-adsorption test has little value when testing affinity purified antibodies.

Comments on paper II: Specificity controls for immunocytochemistry

Background: Still the goal was to quantify and localize EAAC1. To be sure about the specificity of our antibodies when used to label tissue sections we got hold of fixed brains from EAAC1 knockout mouse previously described (Peghini et al., 1997).

Result: This final test of the antibodies against EAAC1gave a surprising result: despite the testing described in Paper I and despite being specific on immunoblotting, the antibodies did label something in the EAAC1 knockout. So instead of getting the data I wanted, I ended up knocking out my own paper. In stead, we summed up all the lessons learnt in this paper.

Discussion: This paper is intended as a guide for immunocytochemistry. Too many papers are published without proper controls, and it is frustrating to see this. Everybody should test their antibodies carefully before they go ahead using them. It is bad to have spent a lot of time on antibodies that are not specific. It is also bad for others that wrong results are published, and it is costly to correct erroneous data published by others. The latter point is illustrated in Paper V.

Comments on paper III: Specificity controls for immunocytochemistry: the antigen pre-adsorption test can lead to inaccurate assessment of antibody specificity

Background: A widely used test for verification of antibody specificity is the pre-adsorption test, in which the antibody is mixed with the antigen used to generate the antibody. If addition of the antigen to the antibody prior to incubation with the sample (tissue sections or Western blots) removes the ability of the antibody to label, then this is taken as proof of specificity. It is, or rather, it should be well known that this test has major limitations as pointed out before (e.g. Pool and Buijs, 1988; Swaab et al., 1977; Burry, 2000; Holmseth et al., 2005; Holmseth et al., 2006). Nevertheless, this test continues to be used, sometimes as the only specificity test. We decided to look a bit more into this and to illustrate the point.

Results: We tested the specificity of several antibodies by using different tests; by performing the antigen pre-adsorption test, by immunoblotting, by using several antibodies to the same antigen, and by using tissue from knockout mice as negative controls. We show that antigen pre-adsorption blocks all binding of the affinity purified

antibodies for the selected antibodies shown here (antibodies to GLT1, EAAC1 and BGT1), regardless of whether this binding is to the transporters under study or to cross-reacting epitopes. Further, we demonstrate that there is no perfect correlation between specificity of labeling seen on immunoblots and labeling seen in sections. This goes both ways. Labeling in sections may be due to cross-reactivity even if the blots look perfect, and labeling in sections may be specific even if the blots are dirty. Extra bands on a blot represent a warning, but are in themselves not an absolute indicator and thereby not a sufficient argument for rejecting a study if there are other reasons to believe that the labeling is specific (e.g. no labeling in sections from a knockout mouse).

Discussion: This shows that the pre-adsorption test does not confirm the identity of the antigens being labeled. Pre-absorbing the antibodies is an alternative to affinity purification in the sense that it shows if the labeling is due to the right antibodies, but this does not tell what the same antibodies bind to in the samples. It can be used as a test when unpurified serum is used because serum contains several antibodies. This test is so widely used that it is important to make people aware of its limitations.

Comments on paper IV: Low density of EAAC1 (EAAT3; slc1a1) glutamate transporters suggests involvement in neuronal metabolism rather than in rapid control of synaptically released glutamate

Background: In papers I-III we have described the difficulties we had with obtaining specific antibodies to EAAC1. The antibodies we had were specific on immunoblots, but when they were tested on tissue sections, they all gave labeling in the knockout. We had no antibody that was suitable for immunocytochemistry. Finally we managed to purify one antibody that was monospecific to EAAC1. This antibody was used to study EAAC1's localization, now with the EAAC1 knockout as control.

Results: This paper consists of four main elements. The first element is the purification of EAAC1. We first tried to purify EAAC1 from brain, but found out that it was easier to

do this from kidney. After having developed a new purification protocol by modifying the procedures used with success for GLAST and GLT1, pure EAAC1 protein was eventually obtained, although in small amounts. The second main element is the determination of EAAC1 concentrations in brain tissue by immunoblotting using known amounts of pure EAAC1 protein as standards. The EAAC1 concentration in the young adult hippocampus was found to be about 100 times lower than that of GLT1. The third element was the generation of a specific EAAC1 antibody to allow localization. The strategy that finally gave rise to good antibodies was somewhat unusual: to grow anaerobic bacteria from rabbits and use extracts from these to absorb and remove unwanted antibodies from antisera prior to affinity isolation on columns with immobilized peptide. EAAC1 was found to be restricted to neuronal cell bodies and dendrites. EAAC1 was not detected in axon-terminals. Most EAAC1 is intracellular. The fourth main element is the determination of the surface area of dendrites and spines, and then to calculate the number of EAAC1 molecules using molecular mass, concentration (gm/liter) and membrane surface area.

Discussion: This paper corrects the literature in several ways. Firstly, we bring to rest the question of whether EAAC1 is expressed in glia or in terminals. Secondly, we address the question of the concentrations and show that there are not enough EAAC1 molecules to capture any significant proportion of released glutamate molecules before they can escape from the synaptic cleft even if the highest EAAC1 concentration should be, as claimed (Conti et al., 1998), in the spine membrane around the postsynaptic density. With a mean maximum density (assuming all EAAC1 is inserted in the membrane) of 90 EAAC1-molecules per μm^2 , and a perisynaptic zone of about 0.2 μm^2 it follows that there may be around 20 EAAC1 molecules per synapse (or six trimers). The number of glutamate molecules released from a synaptic vesicle is believed to be in the range 500-5000. Even if we assume that all of the monomers can bind glutamate, it follows that EAAC1 is able to catch only about 4 % of the glutamate molecules if 500 are released and 0.4 % if 5000 are released. This makes it clear that EAAC1 is expressed at too low levels to make a significant contribution. Further, EAAC1's ultrastructrural localization is uncertain because the post-embedding technique is not sensitive enough. It is also clear

that it is GLT1 that removes most of the synaptic glutamate. The low density of EAAC1 and that it is localized outside the synapses suggest that EAAC1 is not important for removal of extracellular glutamate, but that it may be important for neuronal metabolism because it is the only glutamate transporter expressed in the somato-dendritic compartment of most neurons. This agrees with the claim that EAAC1 is involved in neuronal glutathione synthesis (Aoyama et al., 2008b) as EAAC1 is the primary route for cysteine uptake.

The one uncertainty with this work is whether there are splice variants of EAAC1. We only have antibodies against the C-terminal available. So the immunoprecipitations and the following immunoblotting are both done with a C-terminal antibody. It is then possible that only a fraction of the EAAC1 protein is detected. On the other hand nobody has reported splice variants of EAAC1.

Comments on paper V: A quantitative assessment of glutamate uptake into hippocampal synaptic terminals and astrocytes: New insights into a neuronal role for excitatory amino acid transporter 2 (GLT1)

Background: It was concluded several decades ago that nerve terminals can take up glutamate (for review, see Fonnum, 1984; Ottersen and Storm-Mathisen, 1984; Danbolt, 2001). In fact, many believed that terminals had more uptake activity than glia. This conclusion, however, was weakened by the combination of the findings that GLT1 is present in astrocytes and accounts for 95 % of the total reconstitutable uptake activity (Danbolt et al., 1992), and that glial expression of GLT1 depends on intact nerve terminals (Levy et al., 1995). The presence of GLT1 in terminals was then investigated electron microscopically using antibodies to glutaraldehyde fixed D-aspartate. It was then shown that terminals do have glutamate uptake activity and that this is sodium dependent and is able to concentrate (Gundersen et al., 1993). At this time it was not known that the GLT1 can be selectively inhibited by dihydrokainate and this drug was therefore not tested. However, synaptosome preparations are inhibited by dihydrokainate (Johnston et al., 1979; Robinson et al., 1993), but it could be argued that such preparations are contaminated with glia containing GLT1. And all antibodies that had been made against

GLT1, by several different groups, labeled only glial cells in tissue sections. Another puzzling observation was the high levels of GLT mRNA in some neurons ((Torp et al., 1994; Schmitt et al., 1996; Torp et al., 1997); Berger and Hediger, 1998). Further, the GLT1 knockout mice had virtually no uptake activity (Tanaka et al., 1997). Taken together, this did not add up.

Results: We repeated the electron microscopy experiments by Gundersen and co-workers (1993) and show that the uptake into terminals is sensitive to dihydrokainate like the uptake into astrocytes. Further, we show that nerve terminals in tissue from the GLT1 knockout mice do not have the ability to accumulate D-aspartate. The uptake activity of terminals represents about half of the uptake activity in slices, and about three quarters of that in synaptosome preparations. We did not find any uptake of D-aspartate into spines, the structures that express EAAC1, that is another indication for that EAAC1 does not contribute much to removal of extracellular glutamate. We then noticed that the hippocampal slices have much larger extracellular spaces after *in vitro* incubation than normal perfusion fixed tissue. This meant that the various tissue components are better separated. We made use of this and did post-embedding immunogold for GLT1. Then we managed to show that terminals do express GLT1 protein. Most of the immunoreactivity is found in glial structures (about 80%), but some 5-10% is present in terminals. Some of the immunoreactivity (8%) was found in axons where it was distributed in a plasma membrane surface area several times larger than that of astroglia. This explains why CA3 pyramidal cell bodies have high levels of GLT1 mRNA.

In the mean time others (Chen et al., 2002) claimed that terminals express the b-variant of GLT1. We then showed that the a-variant is the predominant isoform in the terminals, but before we managed to publish the proof, they corrected themselves (Chen et al., 2004) explaining that their antibodies were not specific and that terminals express the a-variant.

Discussion: This work resolves the question of whether the glutamate uptake into nerve terminals is mediated by GLT1 and why CA3 pyramidal cells have GLT1 mRNA. It also support the notion that synaptosome preparations preferentially measure uptake into

terminals. However, the study left us with another question: how can 5-10 % of GLT1 molecules account for half of the uptake activity? This mismatch could be explained by damaged glial cells. Glial cells have a lot of processes and probably many of them will be cut off during the slice-preparation. This explains why the glial cells look collapsed in the slices. The nerve terminals seem to manage the slice-preparation better. They are small, round structures which seem to be well preserved during a slice-experiment. It needs to be done more studies on how the glial cells manage during these slice experiments. It is also a question whether glutamate uptake mediated by GLT1 is differentially regulated in nerve terminals versus glial cells (Xu et al., 2003; Furness et al., 2008). The capacity of the nerve terminal uptake in relation to glial uptake is not known, and we do not know how this uptake is in intact brain. To follow up glial versus nerve terminal glutamate uptake we have floxed the GLT1 gene by adding LoxP-sites to it. In combination with expression of Cre-recombinase this will delete GLT1 in the mouse. The construct works because crossing these animals with Nestin-Cre abolished GLT1 expression in the nervous system.

Comments on paper VI: The concentrations and distributions of three C-terminal variants of the GLT1 (GLT1; slc1a2) glutamate transporter protein in rat brain tissue suggests differential regulation

Background: In paper IV it was confirmed that GLT1a is expressed in nerve terminals, although at low levels. But this paper did not address the question whether other variants could be present in terminals. Quantitative data on protein levels were missing

Results: In this paper we make antibodies to three C-terminal variants of GLT1, and use these antibodies to quantify and localize the variants. To be able to obtain pure variant proteins, cells were transfected with the cDNA of the different proteins. The pure proteins were used as standards for the relative quantifications. Concentrations of total GLT1 protein were normalized using a pan-GLT1 antibody. We find that GLT1a represents about 90 % of total hippocampal GLT1, while GLT1b and GLT1c represented 6 and 1 %, respectively. We also provide the first data on the distribution of the GLT1b

on the subcellular level. The antibodies to GLT1c did give some labeling in tissue sections, but we were unable to detect differences between wild-type and knockout mice. To document the GLT1 distributions in more detail, we also made a web-atlas for GLT1a and –b in collaboration with another group at the Centre for Molecular Biology and Neuroscience. Series throughout a whole rat and mouse brain were cut and labeled with the antibodies. The section were scanned and can be viewed as high-resolution microscopic images showing GLT1 distributions. This is a virtual microscope in a data-repository for online inspection and re-use (http://www.rbwb.org; choose "Atlas of Neurotransporter Distributions").

Discussion:

Here, we addressed the question of whether other GLT1 variants also are expressed in nerve terminals indirectly by comparing the amounts relative to each other. We were not able to detect GLT1b in nerve terminals. Because GLT1b is expressed at levels around 15 times lower than GLT1a, it follows that if GLT1b is in nerve terminals it must be at a level even lower than the GLT1a in terminals. It is valuable to know that the amount of the GLT1b and GLT1c is low in control tissue since it is speculated in that these variants are up-regulated in different neurological diseases. Having splice variants give possibilities for differential regulation and targeting for the different splice variants. We here show that GLT1 splice variants seem to be differentially regulated both during development and in regional expression. The same study should also be repeated on retina since these variants are expressed there.

CONCLUSIONS

The present work demonstrates the importance of proper antibody testing before use (Paper I), and that genetically modified animals is the best negative control for immunocytochemistry (Paper II). The pre-adsorption test is not a proper specificity test as pre-adsorption with the antigen also removes eventually unspecific labeling (Paper III). We show that it is important to determine the quantities of the glutamate transporters to answer whether it is enough of the protein for its proposed functional role. The level of

EAAC1 is about 1% of the GLT1 level. This together with EAAC1's localization outside synapses mean that EAAC1 is unlikely contribute much to synaptic clearance of glutamate and probably plays metabolic roles (Paper IV). GLT1 is the quantitatively dominating glutamate transporter. Although this transporter is predominantly in astrocytes, it is also the transporter responsible for the uptake of glutamate into nerve terminals (Paper V). Nerve terminals express GLT1a. GLT1b is only found in astrocytes. GLT1a represents about 90 % of total hippocampal GLT1, while GLT1b and GLT1c represented 6 and 1 %, respectively (Paper VI).

REFERENCES

1. Aoyama K, Matsumura N, Watabe M, Nakaki T (2008a) Oxidative stress on EAAC1 is involved in MPTP-induced glutathione depletion and motor dysfunction. European Journal of Neuroscience 27:20-30.

2. Aoyama K, Suh SW, Hamby AM, Liu J, Chan WY, Chen Y, Swanson RA (2006) Neuronal glutathione deficiency and age-dependent neurodegeneration in the EAAC1 deficient mouse. Nature Neuroscience 9:119- 126.

3. Aoyama K, Watabe M, Nakaki T (2008b) Regulation of neuronal glutathione synthesis. Journal of Pharmacological Sciences 108:227-238.

4. Arriza JL, Eliasof S, Kavanaugh MP, Amara SG (1997) Excitatory amino acid transporter 5, a retinal glutamate transporter coupled to a chloride conductance. Proceedings of the National Academy of Sciences of the United States of America 94:4155- 4160.

5. Auger C, Attwell D (2000) Fast removal of synaptic glutamate by postsynaptic transporters. Neuron 28:547-558.

6. Bacci A, Sancini G, Verderio C, Armano S, Pravettoni E, Fesce R, Franceschetti S, Matteoli M (2002) Block of glutamate-glutamine cycle between astrocytes and neurons inhibits epileptiform activity in hippocampus. Journal of Neurophysiology 88:2302-2310.

7. Barnett NL, Pow DV (2000) Antisense knockdown of GLAST, a glial glutamate transporter, compromises retinal function. Investigative Ophthalmology and Visual Science 41:585-591.

8. Beart PM, O'Shea RD (2007) Transporters for L-glutamate: An update on their molecular pharmacology and pathological involvement. British Journal of Pharmacology 150:5-17.

9. Beckstrøm H, Julsrud L, Haugeto Ø, Dewar D, Graham DI, Lehre KP, Storm-Mathisen J, Danbolt NC (1999) Interindividual differences in the levels of the glutamate transporters GLAST and GLT, but no clear correlation with Alzheimer's disease. Journal of Neuroscience Research 55:218-229.

10. Berger UV, Hediger MA (1998) Comparative analysis of glutamate transporter expression in rat brain using differential double in situ hybridization. Anatomy and Embryology 198:13-30.

11. Berger UV, Hediger MA (2000) Distribution of the glutamate transporters GLAST and GLT-1 in rat circumventricular organs, meninges and dorsal root ganglia. Journal of Comparative Neurology 421:385-399.

12. Bezzi P, Volterra A (2001) A neuron-glia signalling network in the active brain. Current Opinion in Neurobiology 11:387-394.

13. Bondy SC, Lee DK (1993) Oxidative stress induced by glutamate receptor agonists. Brain Research 610:229-233.

14. Bridges RJ, Kavanaugh MP, Chamberlin AR (1999) A pharmacological review of competitive inhibitors and substrates of high-affinity, sodium-dependent glutamate transport in the central nervous system. Current Pharmaceutical Design 5:363- 379.

15. Burry RW (2000) Specificity controls for immunocytochemical methods. Journal of Histochemistry and Cytochemistry 48:163- 166.

16. Cahoy JD, Emery B, Kaushal A, Foo LC, Zamanian JL, Christopherson KS, Xing Y, Lubischer JL, Krieg PA, Krupenko SA, Thompson WJ, Barres BA (2008) A transcriptome database for astrocytes, neurons, and oligodendrocytes: a new resource for understanding brain development and function. Journal of Neuroscience 28:264-278.

17. Carlsson A, Hansson LO, Waters N, Carlsson ML (1997) Neurotransmitter aberrations in schizophrenia: new perspectives and therapeutic implications. Life Sciences 61:75-94.

18. Chancellor AM, Warlow CP (1992) Adult onset motor neuron disease - worldwide mortality, incidence and distribution since 1950. Journal of Neurology, Neurosurgery and Psychiatry 55:1106-1115.

19. Chaudhry FA, Boulland J-L, Jenstad M, Bredahl MK, Edwards RH (2008) Pharmacology of neurotransmitter transport into secretory vesicles. Handbook of Experimental Pharmacology :77-106.

20. Chaudhry FA, Lehre KP, Campagne MV, Ottersen OP, Danbolt NC, Storm-Mathisen J (1995) Glutamate transporters in glial plasma membranes: highly differentiated localizations revealed by quantitative ultrastructural immunocytochemistry. Neuron 15:711-720.

21. Chen W, Aoki C, Mahadomrongkul V, Gruber CE, Wang GJ, Blitzblau R, Irwin N, Rosenberg PA (2002) Expression of a variant form of the glutamate transporter GLT1 in neuronal cultures and in neurons and astrocytes in the rat brain. Journal of Neuroscience 22:2142-2152.

22. Chen WZ, Mahadomrongkul V, Berger UV, Bassan M, Desilva T, Tanaka K, Irwin N, Aoki C, Rosenberg PA (2004) The glutamate transporter GLT1a is expressed in excitatory axon terminals of mature hippocampal neurons. Journal of Neuroscience 24:1136-1148.

23. Chen YM, Swanson RA (2003) The glutamate transporters EAAT2 and EAAT3 mediate cysteine uptake in cortical neuron cultures. Journal of Neurochemistry 84:1332-1339.

24. Choi DW (1992) Excitotoxic cell death. Journal of Neurobiology 23:1261-1276.

25. Clements JD (1996) Transmitter timecourse in the synaptic cleft: its role in central synaptic function. Trends in Neurosciences 19:163-171.

26. Conti F, DeBiasi S, Minelli A, Rothstein JD, Melone M (1998) EAAC1, a high-affinity glutamate transporter, is localized to astrocytes and GABAergic neurons besides pyramidal cells in the rat cerebral cortex. Cerebral Cortex 8:108- 116.

27. Danbolt NC (2001) Glutamate uptake. Progress in Neurobiology 65:1-105.

28. Danbolt NC, Lehre KP, Dehnes Y, Chaudhry FA, Levy LM (1998) Localization of transporters using transporter-specific antibodies. Methods in Enzymology 296:388-407.

29. Danbolt NC, Storm-Mathisen J, Kanner BI (1992) An [Na^++ K^+]coupled L-glutamate transporter purified from rat brain is located in glial cell processes. Neuroscience 51:295-310.

30. Dehnes Y, Chaudhry FA, Ullensvang K, Lehre KP, Storm-Mathisen J, Danbolt NC (1998) The glutamate transporter EAAT4 in rat cerebellar Purkinje cells: a glutamate-gated chloride channel concentrated near the synapse in parts of the dendritic membrane facing astroglia. Journal of Neuroscience 18:3606-3619.

31. Derouiche A, Rauen T (1995) Coincidence of L-glutamate/L-aspartate transporter (GLAST) and glutamine synthetase (GS) immunoreactions in retinal glia: evidence for coupling of GLAST and GS in transmitter clearance. Journal of Neuroscience Research 42:131-143.

32. Drejer J, Benveniste H, Diemer NH, Schousboe A (1985) Cellular origin of ischemia-induced glutamate release from brain tissue in vivo and in vitro. Journal of Neurochemistry 45:145-151.

33. Dringen R (2000) Metabolism and functions of glutathione in brain. Progress in Neurobiology 62:649- 671.

34. Dunlop J, Lou Z, Zhang Y, McIlvain HB (1999) Inducible expression and pharmacology of the human excitatory amino acid transporter 2 subtype of L-glutamate transporter. British Journal of Pharmacology 128:1485-1490.

35. Eliasof S, Arriza JL, Leighton BH, Amara SG, Kavanaugh MP (1998) Localization and function of five glutamate transporters cloned from the salamander retina. Vision Research 38:1443-1454.

36. Erickson RH, Gum JR jr., Lindstrom MM, McKean D, Kim YS (1995) Regional expression and dietary regulation of rat small intestinal peptide and amino acid transporter mRNAs. Biochemical and Biophysical Research Communications 216:249-257.

37. Fairman WA, Vandenberg RJ, Arriza JL, Kavanaugh MP, Amara SG (1995) An excitatory amino-acid transporter with properties of a ligand-gated chloride channel. Nature 375:599-603.

38. Fonnum F (1984) Glutamate: a neurotransmitter in mammalian brain. Journal of Neurochemistry 42:1-11.

39. Fournier KM, Gonzalez MI, Robinson MB (2004) Rapid trafficking of the neuronal glutamate transporter, EAAC1 - Evidence for distinct trafficking pathways differentially regulated by protein kinase C and platelet-derived growth factor. Journal of Biological Chemistry 279:34505- 34513.

40. Furness D, Dehnes Y, Akhtar A, Rossi D, Hamann M, Grutle N, Gundersen V, Holmseth S, Lehre KP, Ullensvang K, Wojewodzic M, Zhou Y, Attwell D, Danbolt NC (2008) A quantitative assessment of glutamate uptake into hippocampal synaptic terminals and astrocytes: New insights into a neuronal role for excitatory amino acid transporter 2 (EAAT2). Neuroscience 157:80-94.

41. Furness DN, Lehre KP (1997) Immunocytochemical localization of a high- affinity glutamate-aspartate transporter, GLAST, in the rat and guinea-pig cochlea. European Journal of Neuroscience 9:1961-1969.

42. Furuta A, Martin LJ, Lin CL, Dykes-Hoberg M, Rothstein JD (1997a) Cellular and synaptic localization of the neuronal glutamate transporters excitatory amino acid transporter 3 and 4. Neuroscience 81:1031-1042.

43. Furuta A, Rothstein JD, Martin LJ (1997b) Glutamate transporter protein subtypes are expressed differentially during rat CNS development. Journal of Neuroscience 17:8363-8375.

44. Gegelashvili G, Civenni G, Racagni G, Danbolt N, Schousboe I, Schousboe A (1997a) High-affinity glutamate transporters: Molecular diversity and regulatory factors. Journal of Neurochemistry 69:S129.

45. Gegelashvili G, Civenni G, Racagni G, Danbolt NC, Schousboe I, Schousboe A (1996) Glutamate receptor agonists up-regulate glutamate transporter GLAST in astrocytes. Neuroreport 8:261-265.

46. Gegelashvili G, Danbolt NC, Schousboe A (1997b) Neuronal soluble factors differentially regulate the expression of the GLT1 and GLAST glutamate transporters in cultured astroglia. Journal of Neurochemistry 69:2612-2615.

47. Ginsberg SD, Martin LJ, Rothstein JD (1995) Regional deafferentation down-regulates subtypes of glutamate transporter proteins. Journal of Neurochemistry 65:2800-2803.

48. Gonzalez MI, Robinson MB (2004) Neurotransmitter Transporters: Why Dance With

So Many Partners?. Current Opinion in Pharmacology 4:30-35.

49. Grewer C, Rauen T (2005) Electrogenic glutamate transporters in the CNS: molecular mechanism, pre-steady-state kinetics, and their impact on synaptic signaling. Journal of Membrane Biology 203:1-20.

50. Gundersen V, Danbolt NC, Ottersen OP, Storm-Mathisen J (1993) Demonstration of glutamate/aspartate uptake activity in nerve endings by use of antibodies recognizing exogenous D-aspartate. Neuroscience 57:97-111.

51. Hamberger A, Berthold C-H, Karlsson B, Lehmann A, Nystrom B (1983) Extracellular GABA, glutamate and glutamine in vivo - perfusion-dialysis of rabbit hippocampus. Neurology and Neurobiology 7:473-492.

52. Hassel B, Bråthe A (2000) Cerebral metabolism of lactate in vivo: evidence for neuronal pyruvate carboxylation. Journal of Cerebral Blood Flow and Metabolism 20:327-336.

53. Haugeto Ø, Ullensvang K, Levy LM, Chaudhry FA, Honoré T, Nielsen M, Lehre KP, Danbolt NC (1996) Brain glutamate transporter proteins form homomultimers. Journal of Biological Chemistry 271:27715- 27722.

54. Herman MA, Jahr CE (2007) Extracellular glutamate concentration in hippocampal slice. Journal of Neuroscience 27:9736-9741.

55. Hertz L, Dringen R, Schousboe A, Robinson SR (1999) Astrocytes: glutamate producers for neurons. Journal of Neuroscience Research 57:417-428.

56. Himi T, Ikeda M, Yasuhara T, Nishida M, Morita I (2003) Role of neuronal glutamate transporter in the cysteine uptake and intracellular glutathione levels in cultured cortical neurons. Journal of Neural Transmission 110:1337-1348.

57. Holmseth S, Dehnes Y, Bjørnsen LP, Boulland JL, Furness DN, Bergles D, Danbolt NC (2005) Specificity of antibodies: unexpected cross reactivity of antibodies directed against the EAAT3 (EAAC) glutamate transporter. Neuroscience 136:649-660.

58. Holmseth S, Lehre KP, Danbolt NC (2006) Specificity controls for immunocytochemistry. Anatomy and Embryology 211:257- 266.

59. Honig LS, Chambliss DD, Bigio EH, Carroll SL, Elliott JL (2000) Glutamate transporter EAAT2 splice variants occur not only in ALS, but also in AD and controls. Neurology 55:1082-1088.

60. Huggett J, Vaughan-Thomas A, Mason D (2000) The open reading frame of the Na^+-dependent glutamate transporter GLAST-1 is expressed in bone and a splice variant of this molecule is expressed in bone and brain. FEBS Letters 485:13-18.

61. Jackson M, Song W, Liu MY, Jin L, Dykes-Hoberg M, Lin CI, Bowers WJ, Federoff

HJ, Sternweis PC, Rothstein JD (2001) Modulation of the neuronal glutamate transporter EAAT4 by two interacting proteins. Nature 410:89-93.

62. Johnston GA, Kennedy SM, Twitchin B (1979) Action of the neurotoxin kainic acid on high affinity uptake of L-glutamic acid in rat brain slices. Journal of Neurochemistry 32:121-127.

63. Kanai Y, Hediger MA (1992) Primary structure and functional characterization of a high-affinity glutamate transporter. Nature 360:467- 471.

64. Kanner BI, Sharon I (1978) Active transport of L- glutamate by membrane vesicles isolated from rat brain. Biochemistry US 17:3949-3953.

65. Kavanaugh MP, Bendahan A, Zerangue N, Zhang YM, Kanner BI (1997) Mutation of an amino acid residue influencing potassium coupling in the glutamate transporter GLT-1 induces obligate exchange. Journal of Biological Chemistry 272:1703- 1708.

66. Kimelberg HK, Mongin AA (1998) Swelling-activated release of excitatory amino acids in the brain: relevance for pathophysiology. In: Contrib Nephrol. Lang F, eds. Karger, CH-4009 Basel, Switzerland. . .

67. Kiryu S, Yao GL, Morita N, Kato H, Kiyama H (1995) Nerve injury enhances rat neuronal glutamate transporter expression: identification by differential display PCR. Journal of Neuroscience 15:7872-7878.

68. Kiryu-Seo S, Gamo K, Tachibana T, Tanaka K, Kiyama H (2006) Unique anti-apoptotic activity of EAAC1 in injured motor neurons. EMBO Journal 25:3411-3421.

69. Koch HP, Larsson HP (2005) Small-scale molecular motions accomplish glutamate uptake in human glutamate transporters. Journal of Neuroscience 25:1730-1736.

70. Kristensen AS, Geballe MT, Snyder JP, Traynelis SF (2006) Glutamate receptors: variation in structure-function coupling. Trends in Pharmacological Sciences 27:65-69.

71. Kugler P, Schmitt A (1999) Glutamate transporter EAAC1 is expressed in neurons and glial cells in the rat nervous system. Glia 27:129-142.

72. Kugler P, Schmitt A (2003) Complementary neuronal and glial expression of two high-affinity Glutamate transporter GLT1/EAAT2 forms in rat cerebral cortex. Histochemistry and Cell Biology 119:425-435.

73. Laake JH, Slyngstad TA, Haug FM, Ottersen OP (1995) Glutamine from glial cells is essential for the maintenance of the nerve terminal pool of glutamate: immunogold evidence from hippocampal slice cultures. Journal of Neurochemistry 65:871- 881.

74. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

75. Lehre K, Levy L, Chaudhry F, Storm-Mathisen J, Ottersen O, Danbolt N (1993) Localization of glutamate transporters in brain by site-directed immunocytochemistry. Journal of Neurochemistry 61:S251.

76. Lehre KP, Danbolt NC (1998) The number of glutamate transporter subtype molecules at glutamatergic synapses: chemical and stereological quantification in young adult rat brain. Journal of Neuroscience 18:8751-8757.

77. Lehre KP, Levy LM, Ottersen OP, Storm-Mathisen J, Danbolt NC (1995) Differential expression of two glial glutamate transporters in the rat brain: quantitative and immunocytochemical observations. Journal of Neuroscience 15:1835- 1853.

78. Levy LM, Lehre KP, Rolstad B, Danbolt NC (1993) A monoclonal antibody raised against an [Na^+- K^+]coupled L- glutamate transporter purified from rat brain confirms glial cell localization. FEBS Letters 317:79- 84.

79. Levy LM, Lehre KP, Walaas SI, Storm-Mathisen J, Danbolt NC (1995) Down-regulation of glial glutamate transporters after glutamatergic denervation in the rat brain. European Journal of Neuroscience 7:2036-2041.

80. Levy LM, Warr O, Attwell D (1998) Stoichiometry of the glial glutamate transporter GLT-1 expressed inducibly in a chinese hamster ovary cell line selected for low endogenous Na^+-dependent glutamate uptake. Journal of Neuroscience 18:9620-9628.

81. Li S, Mallory M, Alford M, Tanaka S, Masliah E (1997) Glutamate transporter alterations in Alzheimer disease are possibly associated with abnormal APP expression. Journal of Neuropathology and Experimental Neurology 56:901- 911.

82. Lin CL, Orlov I, Ruggiero AM, Dykes-Hoberg M, Lee A, Jackson M, Rothstein JD (2001) Modulation of the neuronal glutamate transporter EAAC1 by the interacting protein GTRAP3-18. Nature 410:84-88.

83. Lipton P (1999) Ischemic cell death in brain neurons. Physiological Reviews 79:1431-1568.

84. Liu MT, Rothstein JD, Gershon MD, Kirchgessner AL (1997) Glutamatergic enteric neurons. Journal of Neuroscience 17:4764- 4784.

85. MacAulay N, Gether U, Klaerke DA, Zeuthen T (2001) Water transport by the human Na^+-coupled glutamate cotransporter expressed in *Xenopus*oocytes. Journal of Physiology - London 530:367-378.

86. Marie H, Billups D, Bedford FK, Dumoulin A, Goyal RK, Longmore GD, Moss SJ, Attwell D (2002) The amino terminus of the glial glutamate transporter GLT-1 interacts with the LIM protein Ajuba. Molecular and Cellular Neurosciences 19:152- 164.

87. Matthews JC, Beveridge MJ, Malandro MS, Rothstein JD, Campbell-Thompson M, Verlander JW, Kilberg MS, Novak DA (1998) Activity and protein localization of

multiple glutamate transporters in gestation day 14 vs. day 20 rat placenta. American Journal of Physiology 274:C603-C614.

88. Melone M, Bellesi M, Conti F (2009) Synaptic localization of GLT-1a in the rat somatic sensory cortex. Glia 57:108-117.

89. Meyer T, Fromm A, Münch C, Schwalenstöcker B, Fray AE, Ince PG, Stamm S, Gron G, Ludolph AC, Shaw PJ (1999) The RNA of the glutamate transporter EAAT2 is variably spliced in amyotrophic lateral sclerosis and normal individuals. Journal of the Neurological Sciences 170:45-50.

90. Meyer T, Münch C, Knappenberger B, Liebau S, Völkel H, Ludolph AC (1998a) Alternative splicing of the glutamate transporter EAAT2 (GLT-1). Neuroscience Letters 241:68-70.

91. Meyer T, Münch C, Liebau S, Fromm A, Schwalenstöcker B, Völkel H, Ludolph AC (1998b) Splicing of the glutamate transporter EAAT2: a candidate gene of amyotrophic lateral sclerosis. Journal of Neurology, Neurosurgery and Psychiatry 65:954.

92. Milton ID, Banner SJ, Ince PG, Piggott NH, Fray AE, Thatcher N, Horne CH, Shaw PJ (1997) Expression of the glial glutamate transporter EAAT2 in the human CNS: an immunohistochemical study. Molecular Brain Research 52:17- 31.

93. Münch C, Schwalenstöcker B, Knappenberger B, Liebau S, Völkel H, Ludolph AC, Meyer T (1998) 5'-heterogeneity of the human excitatory amino acid transporter cDNA EAAT2 (GLT-1). Neuroreport 9:1295-1297.

94. Nicholls D, Attwell D (1990) The release and uptake of excitatory amino acids. Trends in Pharmacological Sciences 11:462-468.

95. Ottersen OP, Laake JH, Reichelt W, Haug FM, Torp R (1996) Ischemic disruption of glutamate homeostasis in brain: quantitative immunocytochemical analysis. Journal of Chemical Neuroanatomy 12:1-14.

96. Ottersen OP, Storm-Mathisen J (1984) Neurons containing or accumulating transmitter amino acids. In: Handbook of Chemical Neuroanatomy: Classical Transmitters and transmitter receptors in the CNS. Bjorklund A, Hökfelt T, and Kuhar MJ, eds. Elsevier Science Publishers B.V., Amsterdam. . .

97. Ottersen OP, Zhang N, Walberg F (1992) Metabolic compartmentation of glutamate and glutamine: morphological evidence obtained by quantitative immunocytochemistry in rat cerebellum. Neuroscience 46:519-534.

98. Owe SG, Marcaggi P, Attwell D (2006) The ionic stoichiometry of the GLAST glutamate transporter in salamander retinal glia. Journal of Physiology 577:591- 599.

99. Peghini P, Janzen J, Stoffel W (1997) Glutamate transporter EAAC-1- deficient mice develop dicarboxylic aminoaciduria and behavioral abnormalities but no

neurodegeneration. EMBO Journal 16:3822-3832.

100. Pines G, Danbolt NC, Bjørås M, Zhang Y, Bendahan A, Eide L, Koepsell H, Storm-Mathisen J, Seeberg E, Kanner BI (1992) Cloning and expression of a rat brain L-glutamate transporter. Nature 360:464-467.

101. Pool CW, Buijs RM (1988) Antigen identity in immunocytochemistry. In: Molecular Neuroanatomy. Van LB, eds. Elsevier Science Publishers B. V. Biomedical Division. . .

102. Rauen T, Wiessner M, Sullivan R, Lee A, Pow DV (2004) A new GLT1 splice variant: cloning and immunolocalization of GLT1c in the mammalian retina and brain. Neurochemistry International 45:1095-1106.

103. Reagan LP, Rosell DR, Wood GE, Spedding M, Munoz C, Rothstein J, Mcewen BS (2004) Chronic restraint stress up-regulates GLT-1 mRNA and protein expression in the rat hippocampus: Reversal by tianeptine. Proceedings of the National Academy of Sciences of the United States of America 101:2179-2184.

104. Reye P, Sullivan R, Scott H, Pow DV (2002) Distribution of two splice variants of the glutamate transporter GLT-1 in rat brain and pituitary. Glia 38:246-255.

105. Robinson MB, Djali S, Buchhalter JR (1993) Inhibition of glutamate uptake with L-trans-pyrrolidine-2,4- dicarboxylate potentiates glutamate toxicity in primary hippocampal cultures. Journal of Neurochemistry 61:2099-2103.

106. Rossi DJ, Oshima T, Attwell D (2000) Glutamate release in severe brain ischaemia is mainly by reversed uptake. Nature 403:316-321.

107. Rothstein JD, Dykes-Hoberg M, Pardo CA, Bristol LA, Jin L, Kuncl RW, Kanai Y, Hediger MA, Wang YF, Schielke JP, Welty DF (1996) Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate. Neuron 16:675-686.

108. Rothstein JD, Martin L, Levey AI, Dykes-Hoberg M, Jin L, Wu D, Nash N, Kuncl RW (1994) Localization of neuronal and glial glutamate transporters. Neuron 13:713-725.

109. Rothstein JD, Patel S, Regan MR, Haenggeli C, Huang YH, Bergles DE, Jin L, Dykes Hoberg M, Vidensky S, Chung DS, Toan SV, Bruijn LI, Su ZZ, Gupta P, Fisher PB (2005) Beta-lactam antibiotics offer neuroprotection by increasing glutamate transporter expression. Nature 433:73- 77.

110. Rothstein JD, Van Kammen M, Levey AI, Martin LJ, Kuncl RW (1995) Selective loss of glial glutamate transporter GLT-1 in amyotrophic lateral sclerosis. Annals of Neurology 38:73-84.

111. Rozyczka J, Engele J (2005) Multiple 5'- splice variants of the rat glutamate

transporter-1. Molecular Brain Research 133:157-161.

112. Rusakov DA, Kullmann DM (1998) Geometric and viscous components of the tortuosity of the extracellular space in the brain. Proceedings of the National Academy of Sciences of the United States of America 95:8975-8980.

113. Ryan RM, Mitrovic AD, Vandenberg RJ (2004) The chloride permeation pathway of a glutamate transporter and its proximity to the glutamate translocation pathway. Journal of Biological Chemistry 279:20742-20751.

114. Sato H, Shiiya A, Kimata M, Maebara K, Tamba M, Sakakura Y, Makino N, Sugiyama F, Yagami K, Moriguchi T, Takahashi S, Bannai S (2005) Redox imbalance in cystine/glutamate transporter-deficient mice. Journal of Biological Chemistry 280:37423-37429.

115. Schlag BD, Vondrasek JR, Munir M, Kalandadze A, Zelenaia OA, Rothstein JD, Robinson MB (1998) Regulation of the glial Na^+-dependent glutamate transporters by cyclic AMP analogs and neurons. Molecular Pharmacology 53:355-369.

116. Schmitt A, Asan E, Lesch KP, Kugler P (2002) A splice variant of glutamate transporter GLT1/EAAT2 expressed in neurons: cloning and localization in rat nervous system. Neuroscience 109:45-61.

117. Schmitt A, Asan E, Puschel B, Jons T, Kugler P (1996) Expression of the glutamate transporter GLT1 in neural cells of the rat central nervous system: non-radioactive in situ hybridization and comparative immunocytochemistry. Neuroscience 71:989- 1004.

118. Schousboe A (1981) Transport and metabolism of glutamate and GABA in neurons and glial cells. International Review of Neurobiology 22:1- 45.

119. Seal RP, Amara SG (1999) Excitatory amino acid transporters: a family in flux. Annual Review of Pharmacology and Toxicology 39:431- 456.

120. Shashidharan P, Huntley GW, Murray JM, Buku A, Moran T, Walsh MJ, Morrison JH, Plaitakis A (1997) Immunohistochemical localization of the neuron-specific glutamate transporter EAAC1 (EAAT3) in rat brain and spinal cord revealed by a novel monoclonal antibody. Brain Research 773:139-148.

121. Shayakul C, Kanai Y, Lee WS, Brown D, Rothstein JD, Hediger MA (1997) Localization of the high-affinity glutamate transporter EAAC1 in rat kidney. American Journal of Physiology 273:F1023-F1029.

122. Shibata T, Yamada K, Watanabe M, Ikenaka K, Wada (1997) Glutamate transporter GLAST is expressed in the radial glia-astrocyte lineage of developing mouse spinal cord. Journal of Neuroscience 17:9212-9219.

123. Shimamoto K, Shigeri Y (2006) Elucidation of Glutamate Transporter Functions Using Selective Inhibitors. :.

124. Shupliakov O, Brodin L, Cullheim S, Ottersen OP, Storm-Mathisen J (1992) Immunogold quantification of glutamate in two types of excitatory synapse with different firing patterns. Journal of Neuroscience 12:3789-3803.

125. Sluse FE (1996) Mitochondrial metabolite carrier family, topology, structure and functional properties: an overview. Acta Biochimica Polonica 43:349- 360.

126. Sontheimer H (2003) Malignant gliomas: perverting glutamate and ion homeostasis for selective advantage. Trends in Neurosciences 26:543-549.

127. Storck T, Schulte S, Hofmann K, Stoffel W (1992) Structure, expression, and functional analysis of a Na^+-dependent glutamate/aspartate transporter from rat brain. Proceedings of the National Academy of Sciences of the United States of America 89:10955-10959.

128. Sullivan R, Rauen T, Fischer F, Wiessner M, Grewer C, Bicho A, Pow DV (2004) Cloning, transport properties, and differential localization of two splice variants of GLT-1 in the rat CNS: Implications for CNS glutamate homeostasis. Glia 45:155-169.

129. Swaab DF, Pool CW, Van Leeuwen FW (1977) Can specificity ever be proved in immunocytochemical staining. Journal of Histochemistry and Cytochemistry 25:388-391.

130. Swanson RA, Chen J, Graham SH (1994) Glucose can fuel glutamate uptake in ischemic brain. Journal of Cerebral Blood Flow and Metabolism 14:1- 6.

131. Swanson RA, Liu J, Miller JW, Rothstein JD, Farrell K, Stein BA, Longuemare MC (1997) Neuronal regulation of glutamate transporter subtype expression in astrocytes. Journal of Neuroscience 17:932-940.

132. Takumi Y, Matsubara A, Danbolt NC, Laake JH, Storm-Mathisen J, Usami S, Shinkawa H, Ottersen OP (1997) Discrete cellular and subcellular localization of glutamine synthetase and the glutamate transporter GLAST in the rat vestibular end organ. Neuroscience 79:1137-1144.

133. Tanaka K (1997) Epilepsy and exacerbation of brain injury in mice lacking glutamate transporter GLT-1 (vol 276, pg 1699, 1997). Science 278:21.

134. Tanaka K, Watase K, Manabe T, Yamada K, Watanabe M, Takahashi K, Iwama H, Nishikawa T, Ichihara N, Hori S, Takimoto M, Wada K (1997) Epilepsy and exacerbation of brain injury in mice lacking the glutamate transporter GLT- 1. Science 276:1699-1702.

135. Torp R, Danbolt NC, Babaie E, Bjørås M, Seeberg E, Storm-Mathisen J, Ottersen OP (1994) Differential expression of two glial glutamate transporters in the rat brain: an in situ hybridization study. European Journal of Neuroscience 6:936-942.

136. Torp R, Hoover F, Danbolt NC, Storm-Mathisen J, Ottersen OP (1997) Differential

distribution of the glutamate transporters GLT1 and rEAAC1 in rat cerebral cortex and thalamus: an in situ hybridization analysis. Anatomy and Embryology 195:317- 326.

137. Trotti D, Rizzini BL, Rossi D, Haugeto Ø, Racagni G, Danbolt NC, Volterra A (1997) Neuronal and glial glutamate transporters possess an SH-based redox regulatory mechanism. European Journal of Neuroscience 9:1236-1243.

138. Trotti D, Volterra A, Lehre KP, Rossi D, Gjesdal O, Racagni G, Danbolt NC (1995) Arachidonic acid inhibits a purified and reconstituted glutamate transporter directly from the water phase and not via the phospholipid membrane. Journal of Biological Chemistry 270:9890-9895.

139. Ullensvang K, Lehre KP, Storm-Mathisen J, Danbolt NC (1997) Differential developmental expression of the two rat brain glutamate transporter proteins GLAST and GLT. European Journal of Neuroscience 9:1646-1655.

140. Utsunomiya-Tate N, Endou H, Kanai Y (1997) Tissue specific variants of glutamate transporter GLT-1. FEBS Letters 416:312-316.

141. Varoqui H, Schäfer MK, Zhu HM, Weihe E, Erickson JD (2002) Identification of the differentiation-associated Na^+/P_Itransporter as a novel vesicular glutamate transporter expressed in a distinct set of glutamatergic synapses. Journal of Neuroscience 22:142-155.

142. Veruki ML, Morkve SH, Hartveit E (2006) Activation of a presynaptic glutamate transporter regulates synaptic transmission through electrical signaling. Nature Neuroscience 9:1388-1396.

143. Volterra A, Bezzi P, Rizzini BL, Trotti D, Ullensvang K, Danbolt NC, Racagni G (1996) The competitive transport inhibitor L-trans-pyrrolidine-2,4- dicarboxylate triggers excitotoxicity in rat cortical neuron- astrocyte co-cultures via glutamate release rather than uptake inhibition. European Journal of Neuroscience 8:2019-2028.

144. Volterra A, Magistretti PJ, and Haydon PG (2002) The Tripartite Synapse - Glia in Synaptic Transmission. Oxford University Press. New York. pp272.

145. Volterra A, Trotti D, Racagni G (1994) Glutamate uptake is inhibited by arachidonic acid and oxygen radicals via two distinct and additive mechanisms. Molecular Pharmacology 46:986-992.

146. Wadiche JI, Arriza JL, Amara SG, Kavanaugh MP (1995) Kinetics of a human glutamate transporter. Neuron 14:1019-1027.

147. Wadiche JI, von Gersdorff H (2006) Long-distance signaling via presynaptic glutamate transporters. Nature Neuroscience 9:1352- 1353.

148. Watabe M, Aoyama K, Nakaki T (2008) A dominant role of GTRAP3-18 in neuronal glutathione synthesis. Journal of Neuroscience 28:9404-9413.

149. Watase K, Hashimoto K, Kano M, Yamada K, Watanabe M, Inoue Y, Okuyama S, Sakagawa T, Ogawa S, Kawashima N, Hori S, Takimoto M, Wada K, Tanaka K (1998) Motor discoordination and increased susceptibility to cerebellar injury in GLAST mutant mice. European Journal of Neuroscience 10:976-988.

150. Waxman EA, Baconguis I, Lynch DR, Robinson MB (2007) N-methyl-D-aspartate receptor-dependent regulation of the glutamate transporter excitatory amino acid carrier 1. Journal of Biological Chemistry 282:17594-17607.

151. Woolley CS, McEwen BS (1992) Estradiol mediates fluctuation in hippocampal synapse density during the estrous cycle in the adult rat. Journal of Neuroscience 12:2549-2554.

152. Xu NJ, Bao L, Fan HP, Bao GB, Pu L, Lu YJ, Wu CF, Zhang X, Pei G (2003) Morphine withdrawal increases glutamate uptake and surface expression of glutamate transporter GLT1 at hippocampal synapses. Journal of Neuroscience 23:4775- 4784.

153. Yamada K, Watanabe M, Shibata T, Tanaka K, Wada K, Inoue Y (1996) EAAT4 is a post-synaptic glutamate transporter at Purkinje cell synapses. Neuroreport 7:2013-2017.

154. Yernool D, Boudker O, Jin Y, Gouaux E (2004) Structure of a glutamate transporter homologue from *Pyrococcus horikoshii*. Nature 431:811-818.

155. Yu AC, Drejer J, Hertz L, Schousboe A (1983) Pyruvate carboxylase activity in primary cultures of astrocytes and neurons. Journal of Neurochemistry 41:1484-1487.

156. Zarbiv R, Grunewald M, Kavanaugh MP, Kanner BI (1998) Cysteine scanning of the surroundings of an alkali-ion binding site of the glutamate transporter GLT-1 reveals a conformationally sensitive residue. Journal of Biological Chemistry 273:14231- 14237.

157. Zerangue N, Arriza JL, Amara SG, Kavanaugh MP (1995) Differential modulation of human glutamate transporter subtypes by arachidonic acid. Journal of Biological Chemistry 270:6433-6435.

158. Zerangue N, Kavanaugh MP (1996a) Flux coupling in a neuronal glutamate transporter. Nature 383:634-637.

159. Zerangue N, Kavanaugh MP (1996b) Interaction of L-cysteine with a human excitatory amino acid transporter. Journal of Physiology - London 493:419- 423.

160. Zhang YM, Bendahan A, Zarbiv R, Kavanaugh MP, Kanner BI (1998) Molecular determinant of ion selectivity of a (Na^++K^+)- coupled rat brain glutamate transporter. Proceedings of the National Academy of Sciences of the United States of America 95:751-755.

I

II

III

Title: Specificity controls for immunocytochemistry: the antigen pre-adsorption test can lead to inaccurate assessment of antibody specificity

Authors: Silvia Holmseth[1], Yun Zhou[1], Virginie V. Follin-Arbelet[1], Knut Petter Lehre[1], Dwight E. Bergles[2] and Niels Christian Danbolt[1]*

Affiliations of all authors:
1 Department of Anatomy, Institute of Basic Medical Sciences, University of Oslo, P. O. Box 1105 Blindern, N 0317 Oslo, Norway.
2 The Solomon H. Snyder Department of Neuroscience, Johns Hopkins University School of Medicine, WBSB 1001, 725 North Wolfe St., Baltimore, MD 21205, USA

Address of corresponding author:
Niels Chr. Danbolt, Centre for Molecular Biology and Neuroscience, Department of Anatomy, Institute of Basic Medical Sciences, University of Oslo, P.O.Box 1105 Blindern, N-0317 Oslo, NORWAY. Fax: (+47) 22 85 12 78, E-mail: n.c.danbolt@medisin.uio.no

ABSTRACT

Most of the high resolution data on the distribution of proteins in tissues have been obtained by means of immunocytochemistry, and the biomedical research community relies directly or indirectly on these data. For instance, interpretation of electrophysiological and pharmacological observations depends on information on where ion channels, receptors, enzymes or transporters are located. Consequently, issues related to immunocytochemical quality control are important. Unfortunately, no single test has sufficient power to unequivocally validate labeling specificity. Several tests have therefore to be used in combination. One test is the "pre-adsorption test". This test was intended for testing crude antisera, but is now frequently used to validate monoclonal and affinity purified polyclonal antibodies. Here we compare the power of this test with that of tests based on tissues from genetically modified animals, multiple antibodies to the same protein, and immunoblots (with or without fixation). We show data from nine different affinity purified antibodies to three proteins (slc1a1, slc1a2, and slc6a12) in combination with genetically modified mice deficient in these proteins. We demonstrate that the pre-adsorption test can be misleading. Further, we show that immunoblotting is more informative, but we also show examples where labeling of immunoblots does not match that seen in sections.

Keywords: Antibody dilution, Glutamate transporter, GABA transporter, Knockout mice, Blot fixation test, Blocking peptide, Neoantigens

INTRODUCTION

Immunochemical techniques have been in widespread use for several decades for identifying individual proteins in complex biological samples (e.g. tissue extracts and sections), and the principles of immunocytochemistry are well established (e.g. Pool and Buijs, 1988). Nevertheless, the field of immunocytochemistry is still troubled by spurious results due to insufficient controls of antibody specificity. Inaccurate immunocytochemical data are a major concern, considering the widespread use of this method and the considerable effort required to correct inaccurate results. Several recent publications have addressed these issues and have proposed guidelines for inclusion of immunocytochemical data (e.g. Saper and Sawchenko, 2003; Saper, 2005; Holmseth et al., 2006; Rhodes and Trimmer, 2006; Lorincz and Nusser, 2008; Fritschy, 2008; Burry, 2011). The arguments for improvements in quality control are strong, but it is hard to define the exact tests that

should be performed. One important step in this direction is to demand detailed descriptions of antibodies (e.g. Saper and Sawchenko, 2003; Saper, 2005; Holmseth et al., 2006; Rhodes and Trimmer, 2006; Fritschy, 2008). Another would be to motivate commercial antibody producers to test their antibodies more rigorously before selling them to scientists who often lack the resources or expertise to evaluate acquired antibodies (Pradidarcheep et al., 2008; Couchman, 2009; Boenisch, 2006; Kalyuzhny, 2009). However, not all testing can be done in advance, because the overall labeling specificity is affected by so many parameters that antibodies have to be tested for each application (e.g: Ottersen, 1987; Holmseth et al., 2006; Lorincz and Nusser, 2008; Rhodes and Trimmer, 2006). Virtually all assay conditions can affect antibody binding, including protein conformation and hydrophobic interactions (e.g. pH, buffer composition and ionic strength), tissue handling steps (e.g. time to fixation, type of fixation, fixative composition, fixation time, storage after fixation) and antigen retrieval techniques (e.g. Josephsen et al., 1999; Willingham, 1999; Burry, 2000; Holmseth et al., 2006; Webster et al., 2009; Boenisch, 2006; Lorincz and Nusser, 2008; Saper, 2009; Hoffman et al., 2010; Paavilainen et al., 2010; Xie et al., 2011).

The scope of the present report is not to provide a comprehensive overview of all aspects of immunocytochemical specificity testing, but to compare the power of the antigen pre-adsorption test with other tests. Antigen pre-adsorption was originally introduced to validate antisera (e.g. Pool and Buijs, 1988; Swaab et al., 1977; Burry, 2000; Burry, 2011), but it is still considered mandatory by many investigators although it is now commonly used to validate labeling obtained with monoclonal or affinity purified antibodies. Here, we tested the specificity of several antibodies (a) by performing the antigen pre-adsorption test, (b) by immunoblotting, (c) by using several antibodies to the same antigen, and (d) by using tissue from knockout mice as negative controls. We show that antigen pre-adsorption blocks all binding of the affinity purified antibodies, regardless of whether this binding is to the proteins under study or to cross-reacting epitopes.

MATERIALS & METHODS

Animals, immunizations and collection of tissue

All animal experimentation was carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996 and the European Communities Council Directive of 24 November 1986 (86/609/EEC). Formal approval to conduct the experiments described was obtained from the animal subjects review board of our institutions. Adult male Wistar rats (10-12 weeks old) were obtained from B&K Universal (Sollentuna, Sweden) and were kept at the animal facility of the Institute of Basic Medical Sciences at the University of Oslo (Oslo, Norway). The C57Black/6 mice (4 weeks old) lacking EAAT3 (Peghini et al., 1997) were raised at the animal facility at the John Hopkins University (Baltimore, USA). The BGT1-fKO mice lacking the BGT1 (slc6a12) gene were in a mixed [C57Blacl/6 x 129] background as described (Lehre et al., 2011), while the EAAT2-KO mice (Tanaka et al., 1997) lacking the EAAT2 gene (GLT1, slc1a2) were in a pure C57Bl/6 background. The mice were kept in the animal facility at the Governmental Institute of Publish Health (Oslo, Norway). The animals (both rats and mice) were killed by lethal injection of pentobarbital and fixed by cardiac perfusion as described previously (Danbolt et al., 1998). New Zealand White rabbits obtained from B&K Universal (Sollentuna, Sweden) were kept in the animal facility at the Institute of Basic Medical Sciences. Rabbits were immunized and bled as described (Danbolt et al., 1998), but using subcutaneous rather than intracutaneous injections.

The three genetically modified mouse lines were maintained by crossing heterozygote mice with each other. This was done to obtain pairs wild-type and knockout mice from the same litters. Thus, potential differences rearing conditions, genetic background and age were thereby minimized.

Ab# id	Purification date	Host species	Antibody name	Animal number	Protein	Antigen peptide name	Antigen sequence	Ligand on affinity column	Specificity
48	1993-06-19	Rb	Anti-B2	81024	EAAT2	B2-11	ASTEGANNMP-(amide)	2-11	EAAT2
360	2002-07-10	Rb	Anti-B12	26970	EAAT2	B12-26	KQVEVRMHDSHLSSE-(amide)	12-26	EAAT2
63	1994-06-05	Rb	Anti-B372	82898	EAAT2	B372-382	RCLEDNLGIDK-(amide)	372-382	EAAT2
95	1994-05-29	Rb	Anti-B493	84946	EAAT2	B493-508	YHLSKSELDTIDSQHR-(amide)	493-508	EAAT2
355	2002-09-05	Rb	Anti-B563	1B0707	EAAT2	B563-573	SVEEEPWKREK-(free acid)	563-573	EAAT2
359	2002-09-19	Rb	Anti-C480	0B0721	EAAT3	C480-499	IVNPFALEPTILDNEDSDTK-(amide)	480-499	EAAT3
371	2003-01-03	Rb	Anti-C491	1B0683	EAAT3	C491-523	CLDNEDSDTKKSYVNGGFSVD KSDTISFTQTSQF-(free acid)	491-523	EAAT3
547	2002-09-19	Rb	Anti-C480-tub	0B0721	EAAT3	C480-499	IVNPFALEPTILDNEDSDTK-(amide)	Tubulin	EAAT3 & Tubulin
565	2005-10-31	Sh	Anti-C510	4131	EAAT3	C491-523	CLDNEDSDTKKSYVNGGFSVD KSDTISFTQTSQF-(free acid)	509-523	EAAT3
323	1999-04-28	Rb	Anti-BGT1(599)	8D0156	BGT1	BGT1(599-614)	SPAKQELIAWEKETHL-(free acid)	599-614	BGT1

Table 1: Antibodies to transporter proteins were made by immunizing animals with synthetic peptides coupled to carrier proteins (keyhole limpet hemocyanin) with glutaraldehyde and then isolating the anti-peptide antibodies by affinity chromatography using columns with immobilized peptide (coupled to N-hydroxysuccinimide activated agarose) as described previously (Danbolt et al., 1998). The antigenic sequences correspond to rat glutamate transporter 2 (EAAT2; NP_058911.2), rat glutamate transporter 3 (EAAT3; NP_037164.3) and mouse betaine-GABA transporter (BGT1; P31651.1). The numbers in the peptide names and in the ligand names correspond to residue numbers in the sequences. The peptides were synthesized as C-terminal amides or free acids as indicated. A cysteine (C) residue was added to the N-terminal of the C491-523 peptide to facilitate directional coupling, but the successful immunizations resulted from coupling by adding glutaraldehyde to a mixture of peptide and carrier protein. Antibody #547 was raised against the C491-523 peptide and then affinity purified using immobilized tubulin.

Antibodies to Excitatory Amino Acid Transporters (EAATs)

The primary antibodies used in the present study are summarized in Table 1. The rabbit antibodies to different parts of rat EAAT2 (Pines et al., 1992) were from the same purified batches as described previously (Furness et al., 2008; Holmseth et al., 2009). The batches of sheep anti-C510 (Ab#565), rabbit anti-C480-Tub (Ab#547) and rabbit anti-C491 (Ab#371) antibodies to rat EAAT3 have also been described (Holmseth et al., 2005). They were made immunizing animals with peptides (Table 1) corresponding to residues 480-499 or 491-523 of rat EAAT3 (NP_037164.3). The antibodies are named after the peptide immobilized on the affinity columns. Thus, the anti-C480 and the anti-C491 antibodies were affinity purified using the same peptide as the animals were immunized with, while the anti-C510 antibodies were collected on a column with a shorter peptide (corresponding to residues 509-523).

When immunizing rabbits with the C480-499 peptide, it was noted (Holmseth et al., 2005) that the ensuing antibodies cross-reacted with tubulin despite absence of primary sequence similarly with EAAT3. The antiserum was passed through a column with glutaraldehyde treated proteins (bovine serum albumin and keyhole limpet hemocyanin) to remove polyreactive antibodies, then through a column containing immobilized tubulin (to remove tubulin-binding antibodies), and finally through a column with immobilized EAAT3 peptide. Antibodies detached from the latter column were devoid of tubulin reactivity (Ab#359, not shown here). The surprise was that the antibodies captured on the column with immobilized tubulin (anti-C480-tub; Ab#547) were able to recognize both the EAAT3 peptide and tubulin (Holmseth et al., 2005).

The rabbit anti-BGT599 (Ab#323) antibodies to BGT1 have not previously been published. They were made in the same way (Danbolt et al., 1998) as the other antibodies, but by immunizing a New Zealand White rabbit (number 8D0156) with a peptide corresponding to residues 599-614 (Table 1) of mouse BGT1 (slc6a12; accession number P31651; Liu et al., 1993) coupled to keyhole limpet hemocyanin with glutaraldehyde. The ensuing antiserum was affinity purified on a column with immobilized BGT599-614 peptide using our standard procedure (Danbolt et al., 1998).

Electrophoresis and immunoblotting

This was done as described (Lehre et al., 1995).

Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto nitrocellulose membranes. Then blots were immunolabeled. Briefly, the blots were washed in phosphate buffered saline, incubated in blocking solution (blocking agent in tris buffered saline) followed by primary antibodies and alkaline phosphatase conjugated secondary antibodies. When not stated otherwise, the blocking agent was 1 % (w/v) bovine serum albumin and 0.05 % (v/v) Tween 20.

When stated, the tissue was homogenized in water and subjected to high speed centrifugation (18000 rpm, 39000 xg, 20 min, 4°C) to separate the water soluble components ("supernatant") from water insoluble ones ("pellet"). The pellets were solubilized in sodium dodecyl sulfate. Brain tissue contains about 100 mg protein per gram wet weight (Lowry et al., 1954). When homogenizing brain tissue in water (S. Holmseth and N.C. Danbolt, unpublished) about half of the proteins will remain in the supernatant which is virtually devoid of integral membrane proteins. Thus, this method is an easy way to increase sensitivity by a factor of about two for detection of transporter proteins.

The blot fixation test

After SDS-PAGE and electroblotting had been done as described above, but before the start of immunolabeling, the blots were washed (1 x 5 min) in 0.1 M sodium phosphate buffer, and then incubated with fixatives of the same composition as used to fix tissue for immunocytochemistry. After fixation, the blots were rinsed (1 x 1 min) in 0.1 M sodium phosphate buffer, incubated (30 min) with 1 M ethanolamine or 2 M Tris-HCl buffer (pH 7.4) to quench aldehyde groups and then immunolabeled as described above.

Light microscopic immunocytochemistry

This was performed exactly as described previously (Holmseth et al., 2009). Briefly, free floating vibratome sections (40 µm thick) were treated with 1 M ethanolamine-HCl (pH 7.4) in sodium phosphate buffer, blocked with 10 % newborn calf serum (NCS) in TBST (300 mM NaCl, 0.5 % Triton X-100 and 100 mM Tris-HCl pH 7.4), and incubated overnight with primary antibodies diluted in blocking solution, followed by secondary antibodies. The alkaline phosphatase procedure used here was chosen because it has relatively low contrast and therefore reveals cross-reactivity better than enhanced chemoluminescence based procedures. The latter procedures typically have higher contrast and therefore give "cleaner" pictures. Omission of the primary antibody led to a virtually complete loss of labeling. This implied both that the secondary antibody did not cross-react with tissue components to any significant degree (Secondary antibody control) and that the tissue or immunoblots did not contain any functional enzymes able to convert the substrates (Label control).

RESULTS

Labeling of tissue sections: the pre-adsorption test versus gene deletion and multiple antibodies

Antibodies to glutamate transporter 2 (EAAT2, slc1a2): Our laboratory has over the years produced 239 different batches of antibodies to EAAT2 using 51 different animals and 46 different synthetic peptides as well as purified EAAT2 protein. For the present study we selected five affinity purified antibodies (see Table 1) already known to recognize the native EAAT2 protein both before and after aldehyde fixation. Here we first tested the selected antibodies on sections from the EAAT2-knockout line (Fig. 1). The assay conditions were deliberately chosen to reveal cross-reactivity (high antibody concentrations and inclusion of Triton X-100 to enhance antibody penetration). As shown (Fig. 1) sections from wild-type mice were strongly labeled. The strong labeling was due to the combination of the labeling conditions, the properties of the antibodies and the fact that EAAT2 is very abundant in the brain representing about 1 % of total brain protein (Lehre and Danbolt, 1998). Under these conditions, only the anti-B372 antibodies produced strong labeling of forebrain sections from the knockout mice. In contrast, the anti-B2 or anti-B12 to the N-terminal part of EAAT2 or anti-B563 to the C-terminus hardly produced any labeling at all in the EAAT2-deficient sections with the exception of the

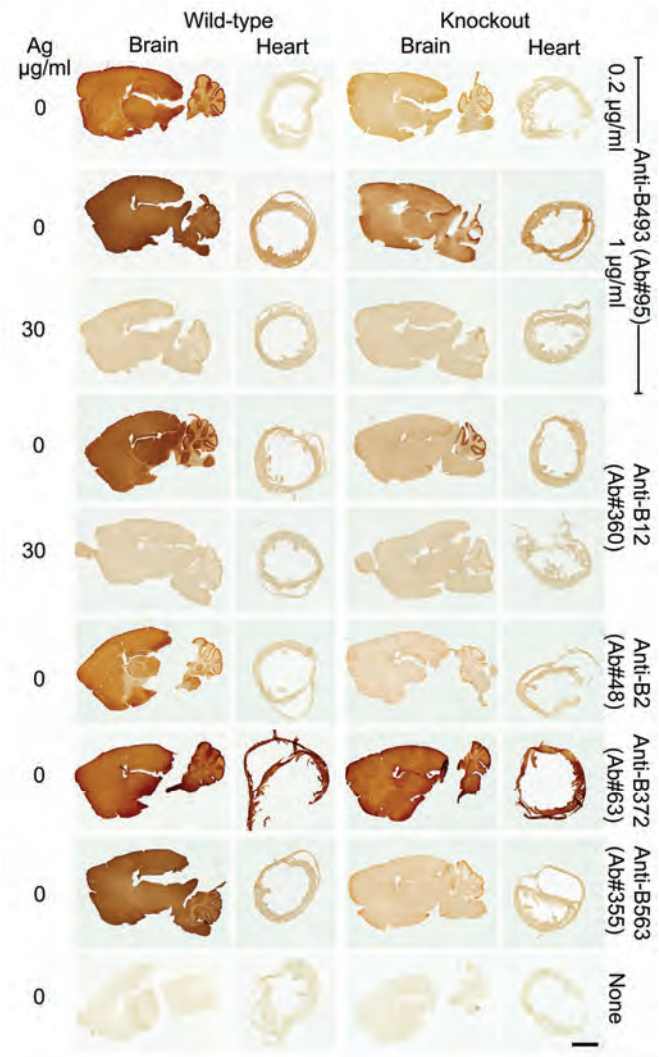


Figure 1: Antibodies (Table 1) to five different EAAT2 epitopes were tested in brain and heart sections from wild-type and EAAT2 knockout mice. Some of the antibodies were pre-adsorbed overnight with 30 µg/ml of their respective peptide antigens (Ag), as indicated, before incubation with the tissue sections. Fixative: the mice were perfusion fixed with 4 % formaldehyde and 0.1 % glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4). Antibody concentrations: anti-B2 (Ab#48) 0.5 µg/ml; anti-B12 (Ab#360) 0.3 µg/ml; anti-B372 (Ab#63) 0.5 µg/ml; anti-B493 (Ab#95) 0.2 µg/ml; anti-B563 (Ab#355) 0.3 µg/ml. Some sections ("None") were developed without primary antibodies to control for the secondary antibodies and the label. Scale bar; 2 mm.

cerebellar molecular layer. This layer was labeled in the sections from the knockout mice with all of the five antibodies, albeit very weakly with anti-B2 and anti-B563. At higher resolution (not shown) it could be seen that there were differences between the antibodies with respect to how they labeled the molecular layer, but all of them labeled Purkinje cells to some degree. The labeling observed in the molecular layer could not be due to EAAT2 because this is a conventional knockout and EAAT2 was completely knocked out in the rest of the brain. Pre-incubation of the antibodies with peptide antigen prior to incubation with the sections eliminated all labeling, whether being specific for GLT1 or representing cross-reactivity.

It should also be pointed out that labeled Purkinje cells are not seen in sections from wild-type animals labeled with anti-B12 (not shown). This is not only because we would normally chose assay conditions that give less strong labeling, but also because of the huge amounts of EAAT2 in glial cells (Lehre and Danbolt, 1998). Thus, the anti-B12 antibodies are sufficiently specific in the adult cerebellum because of the low sensitivity needed to detect EAAT2.

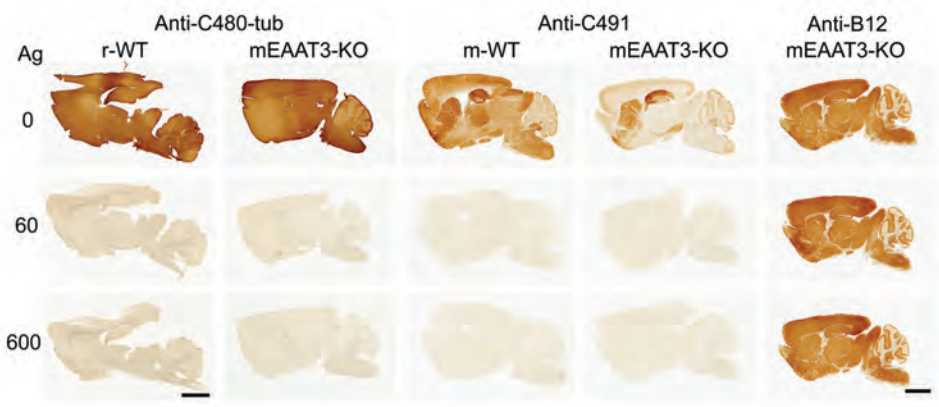


Figure 2: Antigen pre-adsorption blocks all labeling including labeling of cross-reacting epitopes. The same two EAAT3 antibodies as in figure 6, namely anti-C480-tub (Ab#547) and anti-C491 (Ab#371), were pre-absorbed overnight with 0, 60 or 600 µg/ml of the peptide-antigens (Ag) used to generate them (the C480-499 and the C491-523 peptides, respectively) before being used to label tissue sections from wild-type rats (r-WT), wild-type mice (m-WT) and EAAT3 knockout mice (mEAAT3-KO). The fact that the two EAAT3 antibodies label tissue devoid of EAAT3 shows that these antibodies cross-react with non-EAAT3 epitopes. Then, note that pre-adsorption with the peptide-antigens blocks the interaction with *both* the EAAT3 and the non-EAAT3 epitopes. The anti-B12 (Ab#360) antibodies to EAAT2 were pre-absorbed overnight with C491-523 peptide and amounts as indicated. They were unaffected by the presence of EAAT3-peptides (as expected). Also note that the epitope cross-reacting with the anti-C491 antibodies is not uniformly distributed (see also Fig. 3). This shows that an antibody may be specific in one brain region and not in another depending on the distribution of cross-reacting molecules. Scale bar: 4 mm in r-WT and 2 mm in m-WT and m-EAAT3-KO.

Similarly, the antibodies (in particular anti-B493 and anti-B372) labeled heart tissue (cardiomyocytes) while the others only gave weak labeling unless used in high concentrations. Again, the use of multiple antibodies raised the suspicion that the labeling of cardiomyocytes with the anti-B493 and anti-B372 might be due to cross-reactivity. Other data (S. Holmseth and N.C. Danbolt, unpublished) show that the heart does not express EAAT2 (no detectable EAAT2-like uptake activity and the levels of EAAT2 mRNA are exceedingly low). In contrast, pre-adsorption of the anti-B493 antibodies blocked all labeling and gave a misleading impression.

Antibodies to glutamate transporter 3 (EAAT3, slc1a1): Here we compared three of the 87 antibodies we have made (Holmseth et al., 2005) to EAAT3 (anti-C491, anti-C480-tub and anti-C510). The anti-C480-tub antibodies ware produced by fractionation of an antiserum collected after immunization with a peptide representing EAAT3 residues 480-499. These antibodies cross-reacted with tubulin while another fraction from the same serum was highly specific for EAAT3 (Ab#359; not shown here, but see Holmseth et al., 2005). All the antibodies gave rise to labeling in all brain regions in wild-type mice and rats, but anti-C491 and anti-C480-tub also labeled sections from the EAAT3 knockout mice (Fig. 2). The widespread labeling obtained with anti-C480-tub is consistent with the widespread distribution of tubulin. Also the anti-C491 antibodies reacted with the knockout, but in this case the labeling pattern observed in sections from the knockout was not uniform. These antibodies labeled hippocampus CA1-3 and striatum strongly while neocortex and thalamus were virtually unlabeled. This illustrates that cross-reactivity can be highly specific and limited to one tissue or region.

Pre-adsorption of the anti-C491 antibodies with the C491-523 peptide and pre-adsorption of the anti-C480-tub antibody with the C480-499 peptide

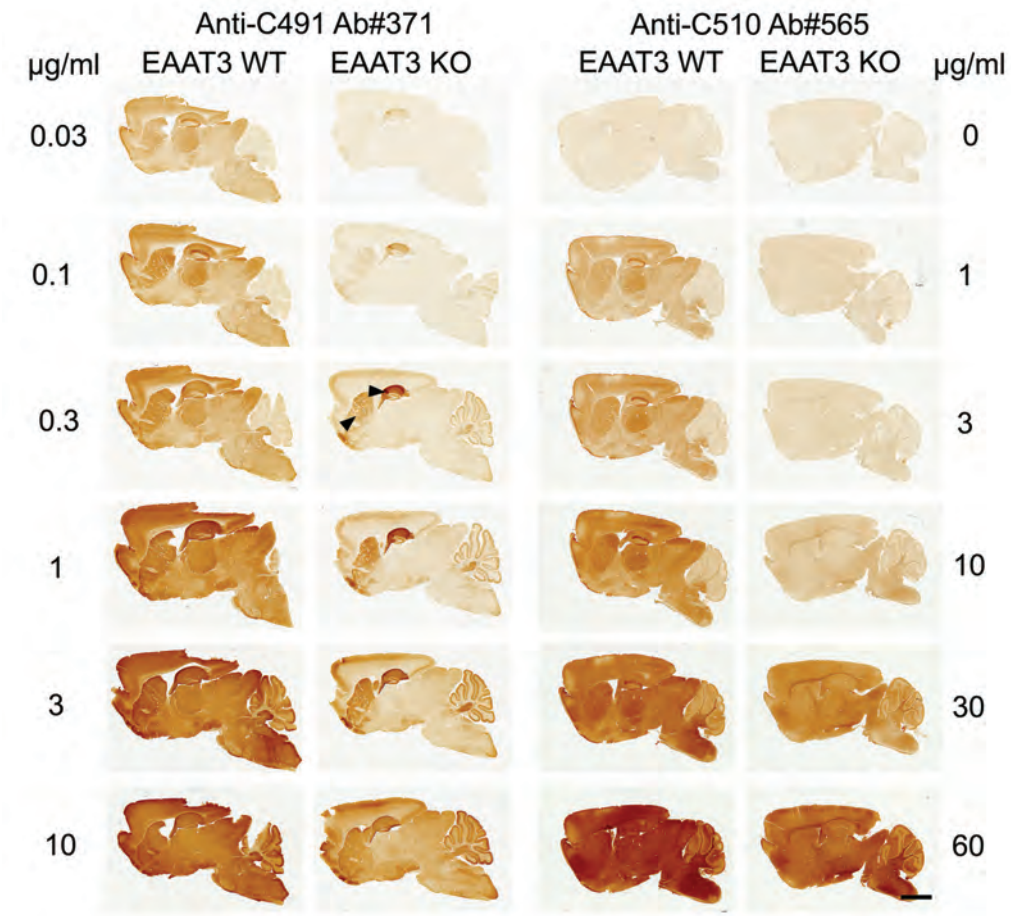


Figure 3: Antibody concentrations affect labeling intensities. Sections from wild-type (WT) and EAAT3-deficient mice (KO) were incubated with either anti-C491 (Ab#371) or anti-C510 (Ab#565) antibodies in concentrations as indicated. Note that anti-C491 cross-react with something else in some regions (arrow heads: hippocampus and striatum). Also note that both antibodies give rise to labeling in all brain regions when used in high concentrations. Consequently, when adjusting labeling conditions (fixation, blocking, antibody concentrations, etc), it is usually possible to get labeling. The difficult part is to determine if the labeling represents the molecule under study or something else. Scale bar: 2 mm.

eliminated all labeling, including labeling of cross-reactive epitopes seen in the sections from the EAAT3-knockout mice (Fig. 2). *Thus, the C480-499 peptide representing EAAT3 blocked interaction between the anti-C480-tub antibodies and tubulin.* Antibodies to EAAT2 were used as a positive control and were unaffected by the addition of EAAT3 peptides.

The immunoreactivity observed in EAAT3 knockout tissue with anti-C491 antibodies raised the question of whether the knockout mice express some EAAT3 protein. This possibility could be ruled out because anti-C510 antibodies at

comparable concentrations did not show this pattern of labeling in sections from the EAAT3 knockout mice (Fig. 3). Figure 3 also illustrates how the labeling depends on the antibody concentrations. Without the negative control that the tissue from the EAAT3 knockout represents, it would have been difficult to discriminate non-specific labeling from that representing EAAT3.

Antibodies to betaine-GABA transporter (BGT1, slc6a12): Our laboratory has made a number of antibodies also to this protein (27 antibody batches, 14 synthetic peptides and 9 animals: S. Holmseth, Y. Zhou and N.C. Danbolt, unpublished). Here we selected one of these antibodies (anti-BGT599). 4G), but not in BGT1 knockout mice (Figs. 4B and 4I). Although this immunoreactivity represents BGT1 (Y. Zhou, S. Holmseth and N.C. Danbolt, unpublished), the most prominent signal obtained This particular antibody to BGT1 labeled tubules in the renal medulla in wild-type mice (Figs. 4A and with this antibody in kidneys was associated with the capillary endothelium, in particular the renal glomeruli in the cortex. Because this labeling was seen in both wild-type (Figs. 4A and 4C) and BGT1 knockout mice (Figs. 4B and 4E), it is highly unlikely to represent BGT1. Further, the other BGT1 antibodies (not shown) did not label endothelium, but they did label medullary tubules. Nevertheless, addition of excess antigen (peptide) to anti-BGT599 led to complete loss of glomerular labeling (Fig. 4D and 4F), indicating that pre-adsorption prevented association with both specific and non-specific epitopes. Also note that there is labeling in the cerebellum of both the wild-type (Fig. 4K) and the knockout (Fig. 4M) mice, but not after antigen pre-adsorption (Fig. 4L and 4N).

The usefulness and limitations of Western blots

Antibodies to BGT1: The anti-BGT1 antibodies were also tested on Western blots. Tissue was collected from both the outer renal medulla and the renal cortex of both wild-type and BGT1 knockout mice. As shown in the left panel of Fig. 5, the BGT1 band was the predominant band (asterisk) and was only observed in the water insoluble fraction from the outer medulla from wild-type mice (Lane 4). Thus, the antibodies appeared to be specific, although some lower molecular mass species were labeled in all lanes containing water insoluble proteins (Lanes 2, 4, 6 and 8) and there was more background in lanes from the cortex (Lanes 5-8) than from the medulla (Lanes 1-4). Consequently, this did not explain the labeling observed in the kidney sections (Fig. 4). There are major differences between sections and Western blots (solubilized molecules may have different conformations and are likely to be separated from their natural molecular neighbors, the smallest and the largest molecules are lost, and the three dimensional structure of the tissue is destroyed). But in addition to this, the tissue used for immunocytochemistry was fixed for one hour in 4 % formaldehyde and then blocked with 10 % newborn calf serum prior to incubation with the antibodies, while the blots were not exposed to aldehydes and were blocked with 1 % bovine serum albumin. To get some assessment of whether fixation and blocking account for these differences, blots were fixed in 4 % formaldehyde for one hour prior to immunolabeling. As shown in Fig. 5 (middle and right panels), a substantial increase in non-BGT1 labeling was observed in this condition. This cross-reactivity was so strong that the BGT1 band was not visible when 1 % bovine serum albumin was used as blocking agent (Fig. 5, middle panel). The stronger blocking (10 % newborn calf serum) used for immunocytochemistry reduced unwanted labeling sufficiently to allow identification of the BGT1 band (Fig. 5, Lane 4 asterisk). Nevertheless, even with this blocking condition, a strongly labeled band (arrow head) appeared in the renal cortex from both wild-type (Lanes 5 and 6) and BGT1 knockout mice (Lanes 7 and 8). This labeling was strong enough to raise legitimate speculation of whether this is the molecular species that give rise to the glomerular labeling in the sections (Fig. 4).

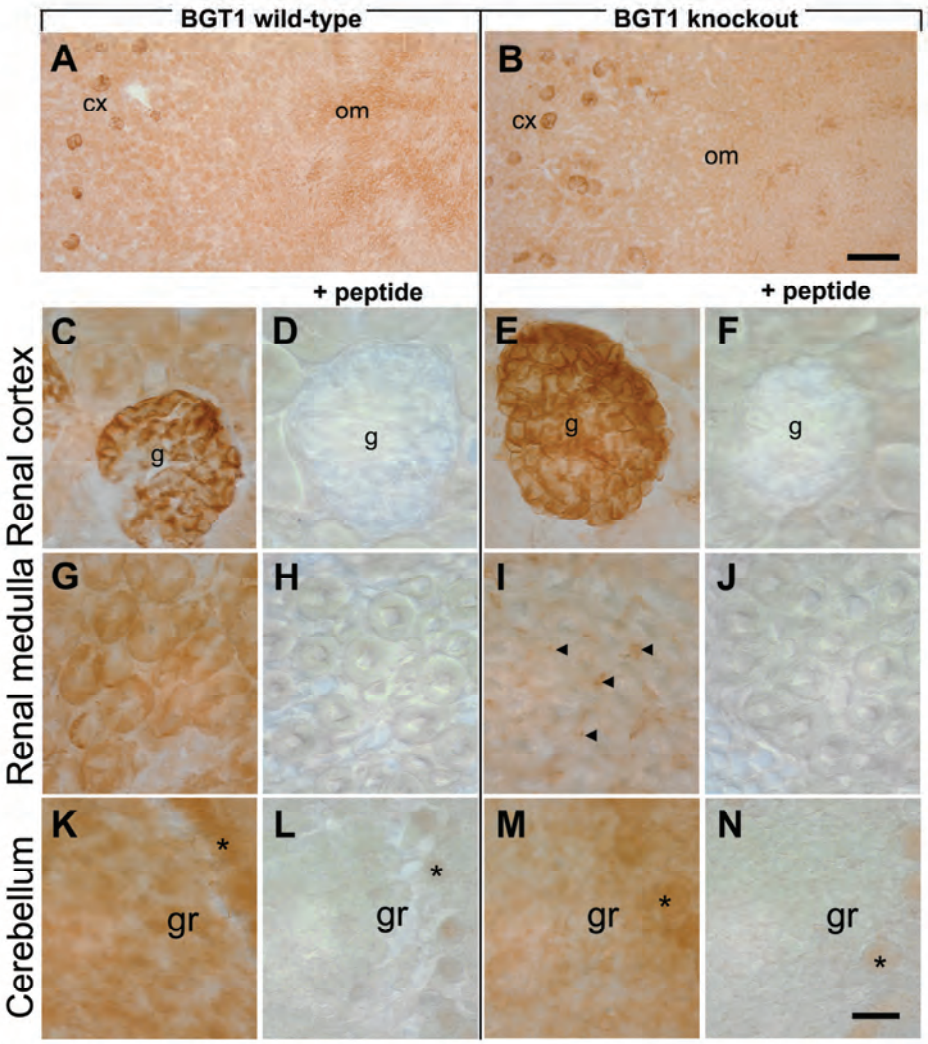


Figure 4: Pre-adsorption test gives a false sense of specificity also in the case of the anti-BGT599 (Ab#323) antibodies to BGT1. The antibodies (3 µg/ml) label the outer renal medulla (om) stronger in wild-type mice (A and G) than in BGT1 knockout mice (B and I). This is the labeling that truly represents BGT1 (Zhou *et al.* 2011), but note labeled glomeruli (g) in the renal cortex (cx) from both wild-type (A, C) and knockout (B, E) mice. This means that the antibody cross-reacts with something present in capillaries in glomeruli and also elsewhere in the kidney as seen in the medulla (I, arrowheads). Also note that there is labeling in the cerebellar granule cell layer (gr) both in wild-type (K) and knockout mice (M). Panels D, H, L, F, J and N show that virtually all labeling, regardless of whether it represents BGT1 or not, is abolished if the antibodies are pre-adsorbed with the peptide (1 mg/ml BGT599-614) to which they have been raised and affinity purified. The only remaining labeling (*) seen in panels L and N is due to reactivity of the secondary antibody. Fixative: 4 % formaldehyde without glutaraldehyde. Scale bars: A and B, 200 µm; C-N, 20 µm.

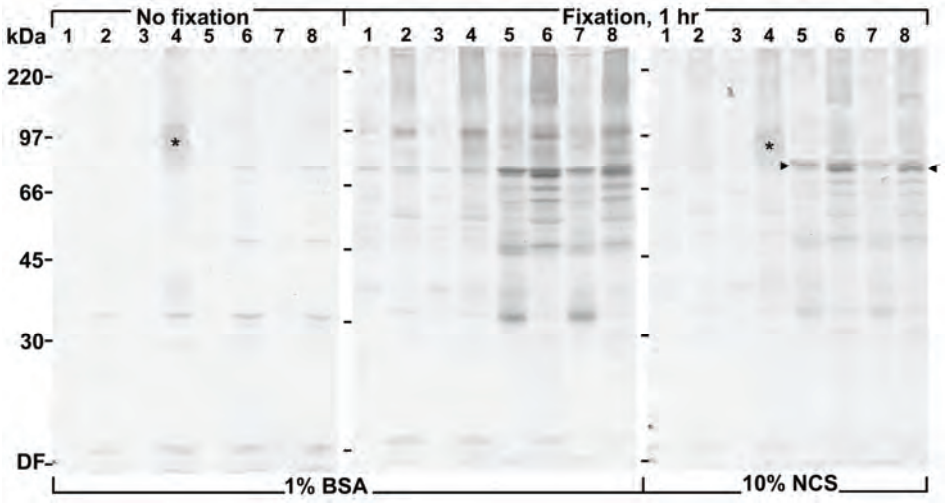


Figure 5: Fixation creates new epitopes cross-reacting with the anti-BGT1 antibodies. Tissue from the renal medulla (Lanes 1-4) and cortex (Lanes 5-8) from both BGT1 knockout (Lanes 1, 2, 5 and 6) and wild-type (Lanes 3, 4, 7 and 8) mice was homogenized in water to yield water soluble (Lanes 1, 3, 5 and 7) and water insoluble fractions containing membrane proteins (Lanes 2, 4, 6 and 8). Three identical blots were made. Before development with the anti-BGT1 antibodies (Ab#323; 0.5 µg/ml), two of the blots (as indicated: middle and right) were incubated (1h, room temperature) in the same fixative as was used to fix the tissue for immunocytochemistry (Fig. 4). The unfixed (left) and one of the fixed blots (middle) were developed using 1 % bovine serum albumin (BSA) as blocking agent, while the last was blocked with the same blocking agent as was used for immunocytochemistry (Fig. 4: 10 % newborn calf serum (NCS)). The band representing the BGT1 protein is indicated (Lane 4; asterisk). Note that many extra bands are seen after fixation (arrowheads).

Antibodies to EAAT3: The anti-C491 antibodies recognized EAAT3 in brain extracts from both rat and wild-type mice, but not from EAAT3 knockout mice (Fig. 6A). This antibody looked specific when tested on Western blots and the blots therefore did not explain the cross-reactivity observed in sections (Figs. 2 and 3). Brains from wild-type and EAAT3 knockout mice were dissected to enable blotting (Fig. 6CD) of hippocampus, neocortex, and cerebellum separately. Further, 10-20 % gradient gels were used to detect more of the smaller proteins. Two identical blots were made. Fixation of one of the blots (6D) showed that fixation enhanced binding to cross-reactive epitopes, but there was in this case no clear correlation between blots and sections. While the immunocytochemistry showed particularly strong cross-reactivity in the hippocampus, the blots did not reveal any hippocampus specific cross-reactive molecular species.

The anti-C480-tub antibodies (Fig. 6B) recognized a band (arrowhead) with slightly lower apparent molecular mass than EAAT3. This band was present in all the three lanes, including the one with the extract from the EAAT3 knockout, in agreement with the conclusion (Holmseth et al., 2005) that this antibody recognizes tubulin. This band became visible after very short development times, while longer development times were needed to visualize EAAT3. The arrow in Fig. 6B points to the location of EAAT3. The reason is that tubulin is one of the most abundant proteins in the brain (Shelanski et al., 1973) while EAAT3 represents less than 0.01 % (Danbolt et al., 2006). Thus, the labeling of this blot matches the labeling seen in the sections.

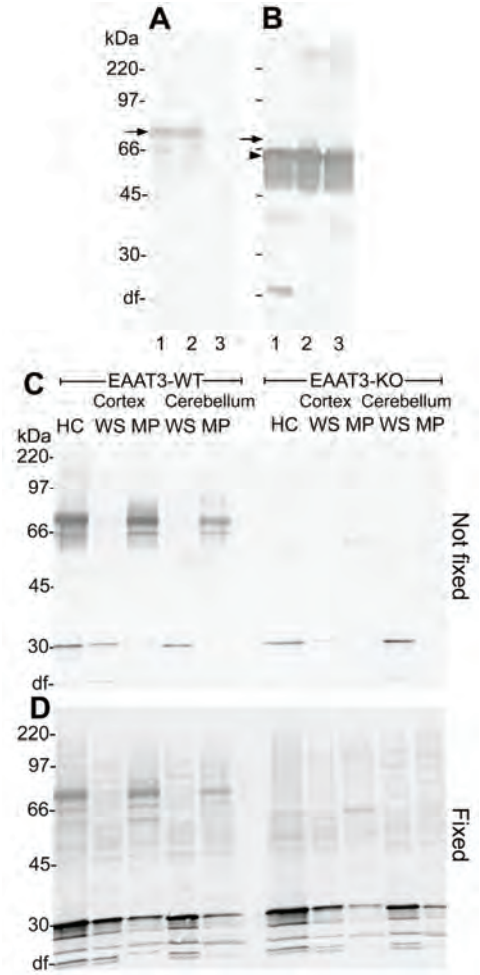


Figure 6: Immunoblots showing the specificity of two different antibodies to EAAT3. (**A, C and D**) anti-C491 (Ab#371; 0.5 µg/ml) and (**B**) Anti-C480-tub (Ab#547; 1 µg/ml). Each lane contained 20 µg of SDS-extracted whole hippocampal tissue proteins obtained from wild-type Wistar rats (Lane 1), wild-type mice (Lane 2) and EAAT3 knockout mice (Lane 3). Note that the anti-C491 antibodies clearly visualize the EAAT3 protein (arrow) in extracts from wild-type mice and rats (Panel A, Lanes 1 and 2). There is no labeling of proteins from the EAAT3 knockout (Panel A, Lane 3). In contrast, the anti-C480-tub antibodies label the EAAT3-band weakly (too weak to be easily seen on this blot) (arrow), but they label another protein (tubulin) strongly (arrowhead). This protein is present in very high concentrations in all the three protein extracts (Panel B, Lanes 1, 2 and 3). Note that the labeling has reached saturation. The identities of the lower bands have not been determined, but may represent partly proteolysed tubulin. Panels C and D represent two identical blots of 10-20 % gradient gels. Gradient gels were used to detect also low molecular mass proteins. The blot in C is unfixed while the blot in D was fixed before incubation with the antibodies The blots contain extracts from mouse hippocampus (HC, whole tissue), mouse neocortex (WS, water soluble; MP, membrane pellet), and mouse cerebellum (WS, water soluble; MP, membrane pellet) separately.

Testing of antibodies to EAAT2: As shown (Fig. 7) all of the antibodies used in figure 1 recognized EAAT2 in brains from wild-type mice. There was strong labeling of a band at around 70 kDa (Lane 1), and this band is not seen in brain samples from the EAAT2-knockout mice (Lane 2). The anti-B493 antibodies also labeled a band with lower molecular mass (below the 45 kDa marker) fairly strongly. This may explain why this antibody gave some labeling of brain tissue from the knockout mice. The rest of the antibodies, however, look highly specific on blots of forebrain (Fig. 7) and cerebellum (not shown). Thus, in the case of anti-B2, anti-B12, anti-B493 and anti-B563 there are good correlations between the labeling seen on blots and in sections. These four antibodies did not give much labeling in sections from the knockout mice (Fig. 1). In contrast, although the anti-B372 antibodies looked highly specific on blots, they still labeled tissue from the knockout mice strongly (Fig. 1). Further, note that anti-B493 and the anti-B2 antibodies labeled bands (Fig. 7, arrowheads) that could be mistaken for EAAT2 in the heart samples. The presence of these bands in the sample from the knockout, and the fact that the other antibodies did not label them, proved that this was not EAAT2. In contrast, pre-adsorbing the anti-B493 with the peptide blocked the labeling giving the mistaken impression that the labeling was specific for EAAT2. Fixing the blots before immunolabeling gave more information. The labeling obtained with the anti-B12 and the anti-B563 antibodies appeared virtually unaffected. However, fixation created several epitopes that cross-reacted with the anti-B493, the anti-B2 and the anti-B372. This may, at least in part, explain why anti-B493 and anti-B372 labeled cardiomyocytes

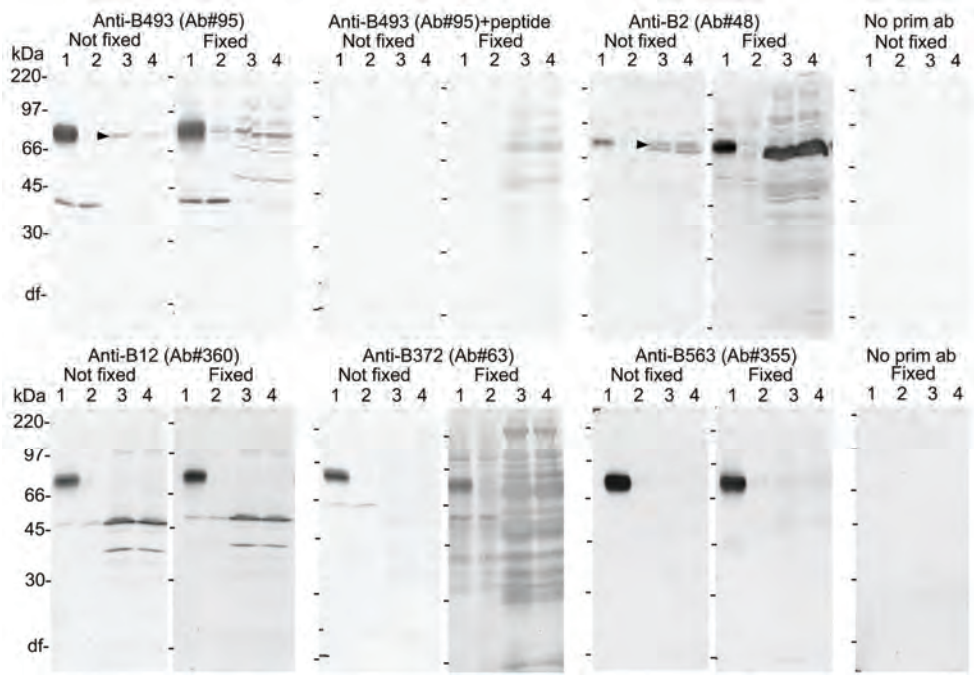


Figure 7: Forebrain (Lanes 1 and 2) and heart (Lanes 3 and 4) tissue from wild-type (Lanes 1 and 3) and EAAT2 knockout (Lanes 2 and 4) mice were solubilized and immunoblotted with the same antibodies were as used in Fig. 1. Fourteen identical blots were prepared. Half of them were fixed (3 h, 4 % formaldehyde and 0.1 % glutaraldehyde in 0.1 M NaPi) before being developed with the antibodies as indicated. One of the antibodies, anti-B493 (Ab#95), were pre-adsorbed overnight with 10 µg/ml peptide-antigen (Ag). This low antigen concentration was sufficient to abolish both the labeling representing EAAT2 and the cross-reactivity seen on unfixed blots. Two of the antibodies (anti-B2 and anti-B493) label heart (arrowheads) at the same molecular mass as EAAT2 (Lane 1), but note that this band is also seen in the knockout (Lane 4). Also note that the anti-B2 antibodies reacted better with the fixed EAAT2 than the unfixed protein, while other antibodies showed weaker reaction after blot fixation (not shown). Antibody concentrations: anti-B2 (Ab#48) 1 µg/ml; anti-B12 (Ab#360) 0.2 µg/ml; anti-B372 (Ab#63) 1 µg/ml; anti-B493 (Ab#95) 0.2 µg/ml; anti-B563 (Ab#355) 0.2 µg/ml. The lanes contained either 5 µg of forebrain protein or 20 µg heart protein extracted with SDS.

(Fig. 1). But also note that the cross-reaction observed with the anti-B2 antibodies on blots of heart proteins did not occur in sections as the latter were unlabeled (Fig. 1).

DISCUSSION

Burry (2011) introduced a new classification of immunocytochemical controls. According to this system there are three groups of controls: primary antibody controls, secondary antibody controls and label controls. The focus of the present study is primary antibody controls which are usually the most difficult ones.

Specificity and sensitivity

The labeling is sensitive if low concentrations of the antibodies give rise to labeling, and it is specific if the antibodies bind only to the target antigen. At low antibody concentrations, only the antigens that have the highest affinities will be labeled. Antigens interacting with lower affinities will be labeled at higher antibody concentrations. Thus, if the antigen of interest binds the antibodies with much higher affinity than other antigens, then the labeling will be specific at low concentrations. One problem is that cross-reactive antigens can sometimes bind with high affinity as illustrated here and by others (e.g.

Josephsen et al., 1999; Dolman et al., 2004; Holmseth et al., 2005; Lorincz and Nusser, 2008). Another problem is that illustrated in figure 3. Labeling can usually be obtained by adjusting the assay conditions to increase sensitivity. The labeling of sections from knockout mice mimics situations where a protein is not present or present below detection limit. It is not easy to distinguish a true positive signal from a false positive signal. Several antibodies to the same protein can help, but as shown with the selected antibodies to EAAT2 (Fig. 1), several antibodies may have the same reactivity. In particular, we have frequently observed cross-reactivity with mitochondria, post-synaptic densities, cell nuclei and the cerebellar molecular layer. Thus, immunocytochemical labeling is not in itself a proof of expression. Consequently, to avoid chasing "ghosts" it is imperative to use other methods (e.g. immunoblots, mRNA quantifications, enzyme activity or other relevant parameters) to verify that the target antigen is indeed present at physiological relevant concentrations in the tissue under study.

The psychology

The start of an immunocytochemistry project is usually an intriguing hypothesis predicting the presence of the protein. And if beautiful images are obtained, everyone gets optimistic. The labeling observed in the sections from the knockout mice shown here could easily have been interpreted as true labeling. And in the case of the series of EAAT2 antibodies, the weaker labeling with the anti-B2 and anti-B563 antibodies could have been disregarded by arguing that these are splice variant specific antibodies. This argument might even have made the hypothesis more interesting. The pre-adsorption test would have supported the exciting interpretation. Thus, the pre-adsorption test would have been dangerously misleading.

Why it is difficult to verify labeling specificity

Labeling is assumed to be specific when all control experiments have failed to detect cross-reactivity. This perspective must be kept in mind when designing and interpreting control experiments. As pointed out (Saper and Sawchenko, 2003; Holmseth et al., 2005), an antibody molecule is not a "magic bullet" with absolute specificity, but a protein molecule which recognizes the antigen much like a receptor protein recognizes the ligand or an enzyme recognizes the substrate. Antibodies can adhere to other molecules, according to their respective concentrations and affinities (Rhodes and Trimmer, 2006; Fritschy, 2008). A good antibody binds to the desired target with high affinity, allowing it to be used at a concentration well below the concentration where it starts to bind to other targets. It should be recalled how medicinal chemists are able to develop new molecules that bind to the same receptor as endogenous ligand in spite of having a very different chemical structure. From this perspective it is not surprising that antibodies often cross-react with seemingly unrelated molecules. It should also be taken into account that tissue processing (post mortem delay, fixation, embedding and antigen retrieval) chemically modifies the tissue, leading to the creation and elimination of epitopes (e.g. Josephsen et al., 1999).

Poly-reactive antibodies are common

Since we made the first antibodies to a glutamate transporter (Danbolt et al., 1992), our laboratory has produced about one thousand different antibodies, only a minority of which lacked non-specific labeling. Even after affinity purification, most antibodies obtained from immunizing rabbits still displayed some degree of cross-reactivity with unrelated proteins (Holmseth et al., 2005). Our impression is that this problem gets worse if the animals are given strong adjuvants that trigger tissue damage around the injection sites, possibly because this may contribute to production of auto-antibodies, e.g. lupus erythematosus-like anti-DNA antibodies. Many of these antibodies do not only bind to DNA, but also to peptide sequences (Sibille et al., 1997; James et al., 1999) and may even cross-react with the glutamate receptor subtype NR2 (DeGiorgio et al., 2001).

Thus, it is important to realize that cross-reactivity does not have to be due to the presence of contaminating antibodies, but can be due to the

antigen recognizing antibodies themselves, as shown here with the anti-C480-tub antibodies and shown previously with monoclonal antibodies (Danbolt et al., 1998). During the process of generating monoclonal antibodies to the EAAT2, several monoclonal antibodies turned out to recognize multiple bands on Western blots.

The correct use of the pre-adsorption control

In the early days of immunocytochemistry, only crude sera were used to label sections. A serum contains a huge number of different antibodies. Most of these are produced by the host animal so that it can protect itself against infections. Experimental immunizations with the antigen of interest, will cause changes in the antibody expression profile and, hopefully, but not necessarily, to production of new antibodies specifically recognizing the antigen to be studied. The content of total IgG in rabbit sera is about 10 mg/ml, but only a small fraction of these (may be a couple of percent in a good antiserum) will be antibodies to the injected antigen. Therefore, when a serum is used for labeling of tissue sections, it is important to determine if the labeling is due to antibodies directed toward the antigen or to other antibodies. A first indication can be obtained by comparing serum collected after immunization (immune-serum) with serum collected before immunization (pre-immune serum). This tells if the immunoreactivity of the serum was there before immunization started or came after the immunization (and therefore may be a consequence of it), but this does not tell if the labeling is due to antibodies to the target antigen or to antibodies to other substances (e.g. components of the adjuvant used to enhance the immuneresponse). This is where the pre-adsorption control comes in. If addition of the target antigen to the serum prevents the serum from labeling the sections, then it follows that the labeling is due to those antibodies that are able to bind the added antigen. It is important to note that this does not test if the antibodies also can bind other antigens. For example, assume that the purpose of an experiment is to localize antigen X, and that the available antiserum contains both antibodies to this antigen and multiple different antibodies to other unknown antigens (collectively referred to as non-X). Then assume that the antibodies to X cross-react with another non-X antigen (Y). If pre-adsorption with X does not block the labeling, then the labeling cannot represent X because it must be due to non-anti-X antibodies. On the other hand, if addition of X does indeed block all labeling, then the labeling must be due to the anti-X antibodies and not to any of the other antibodies to non-X. This would be a good sign in that this means that the labeling is due to anti-X antibodies. However, the anti-X antibodies are in this case also able to bind Y, and pre-adsorption with X will block labeling to both X and Y. Thus, the pre-adsorption will tell that anti-X is responsible, but not if anti-X binds to X or to Y or to both in the sections.

Thus, when crude sera are being used, then the pre-adsorption test does add valuable information. If the antigen can be readily obtained in pure form, and in particular if tissue from knockout animals are unavailable, this test should be carried out. A common situation today, however, is to work with antibodies that are already selected for their ability to bind to the antigen (monoclonal or affinity purified polyclonal antibodies). In this situation, we do not need the pre-adsorption test to tell us that the antibodies we are working with have the ability to interact with the antigen. Possible binding of the antibodies via a mechanism not involving antigen binding sites (e.g. by inadequate blocking of unspecific binding or presence of antibody binding molecules such as bacteria expressing protein A) is likely to be revealed by using normal antibodies (e.g. pre-immune antibodies) so the pre-adsorption test is not relevant for that either.

Notes on immunoblots

As explained above, the antibodies shown here have not been randomly selected, but been selected to illustrate a number of points. Together they may give the impression that there is poor correlation between labeling of immunoblots and sections. But that is not the message we want to send. Our experience with immunoblots is that they are informative. They are also relatively inexpensive. If

antibodies look specific on blots, then they are often also specific in sections. Nevertheless, exceptions like those shown here are so common that immunoblots should be supplemented with other tests whenever possible.

When interpreting immunoblots, it is important to keep in mind that one band may contain more than one protein. The anti-B493 antibodies (Fig. 7) illustrate this point. A weak non-EAAT2 band is present in Lane 2 (brain from the knockout mice) as well as in the heart samples (arrowhead). The most likely interpretation is therefore that the EAAT2 band in lane 1 is a mixture of (mostly) EAAT2 and this other molecular species.

Another point to keep in mind is that one protein may give rise to several bands by a variety of mechanisms (e.g. partial proteolysis, post translational modifications, or oligomerization). Extra bands are frequently observed, but it is often hard to determine the identity of them. In the present study this is fairly straight forward because samples from knockout mice were run as controls, and because we have access to several antibodies to each protein. Bands present in the samples from the knockout mice must represent cross-reacting molecules. Further, if antibodies to several parts of the same protein are available [in our case we have antibodies to nine different regions of EAAT2 as well as to the various splice variants (Holmseth et al., 2005, 2009)] then extra bands will be labeled by several antibodies if they indeed represent the same protein. However, as we have pointed out above, bands representing cross-reactivity are so common that our advice is to assume that extra bands represent cross-reactivity unless positive evidence can be provided.

Genetically modified tissue

As pointed out by many (e.g. Holmseth et al., 2006; Burry, 2011), not even genetically modified organisms are perfect specificity controls. For instance, when a gene is deleted, then this may affect expression of other genes. Cross-reactive molecules may be down-regulated or up-regulated. Nevertheless, as illustrated here, knockout animals do represent very powerful controls. The main problem is their availability and the fact that most of them are mice while most immunocytochemistry is done on rat and human tissue. Another problem can be other genes containing the same sequence, or residual expression of the deleted gene unless the deleted sequence includes the part containing the epitope or care has been taken to carry out the deletion in such way that it causes a shift of the reading frame. However, if an antibody gives rise to labeling in knockout tissue, then this should not be taken lightly. Unless residual expression can be proven, then it is our experience that the most likely explanation is unwanted reactivity of the antibodies. We have tested a fairly large number of antibodies on tissue from seven different knockout lines (those shown here and unpublished data from antibodies to slc1a3, slc6a1, slc6a11 and sc6a13).

Another problem with transgenic animals is the costs limiting the number of laboratories that can afford it. But although testing on tissue from knockout animals is expensive in the short term, this may be the cheapest for the society as a whole in the long run because the most expensive is to work on a project for long time and then discover that the labeling is insufficiently specific or even publish erroneous data. Wen incorrect data are published, correction is even more costly.

Conclusions

The primary focus of this study has been to evaluate the pre-adsorption test. We rediscover and illustrate that this test is not a specificity test in the true sense, but test to identify the population of antibodies that is responsible for the labeling. This test does not tell if the observed labeling represents a specific visualization of the antigen under study or if it is due to cross-reaction with other molecules. This is old knowledge and has been discussed, albeit not illustrated, in several publications (e.g. Swaab et al., 1977; Pool and Buijs, 1988; Burry, 2000; Holmseth et al., 2005; Fritschy, 2008; Burry, 2011). Despite this, the pre-adsorption test is still regarded by many as an obligate control for the verification of immunocytochemical labeling - even labeling obtained with monoclonal and affinity purified

antibodies. As shown here the pre-adsorption test can give a misleading impression of specificity. Compounding this problem, it is often costly to obtain enough free antigen to perform the test, diverting time and resources from more definitive experiments.

Acknowledgements: We thank Henriette Danbolt for technical assistance. This work was supported by the Norwegian Research Council (FUGE II-183727-S10) and by the Norwegian Advanced Research Program (Toppforskningsprogrammet).

REFERENCES

Boenisch T. 2006. Heat-induced antigen retrieval: what are we retrieving?. J Histochem Cytochem 54:961- 964.

Burry RW. 2000. Specificity controls for immunocytochemical methods. J Histochem Cytochem 48:163- 166.

Burry RW. 2011. Controls for immunocytochemistry: an update. J Histochem Cytochem 59:6-12.

Couchman JR. 2009. Commercial antibodies: the good, bad, and really ugly. J Histochem Cytochem 57:7-8.

Danbolt NC, Furness DN, Dehnes Y, Qureshi A, Rossi D, Hamann M, Grutle NJ, Gundersen V, Holmseth S, Lehre KP, Ullensvang K, Wojewodzic M, Attwell D. 2006. A quantitative assessment of glutamate uptake into hippocampal synaptic terminals and astrocytes. :.

Danbolt NC, Lehre KP, Dehnes Y, Chaudhry FA, Levy LM. 1998. Localization of transporters using transporter-specific antibodies. Methods Enzymol 296:388-407.

Danbolt NC, Storm-Mathisen J, Kanner BI. 1992. An [Na^++ K^+]coupled L-glutamate transporter purified from rat brain is located in glial cell processes. Neuroscience 51:295-310.

DeGiorgio LA, Konstantinov KN, Lee SC, Hardin JA, Volpe BT, Diamond B. 2001. A subset of lupus anti-DNA antibodies cross-reacts with the NR2 glutamate receptor in systemic lupus erythematosus. Nat Med 7:1189-1193.

Dolman DE, Lawrenson JG, Abbott NJ. 2004. Cross reactivity of polyclonal GFAP antiserum: implications for the in- vitro characterisation of brain endothelium. Brain Res 1012:185- 186.

Fritschy J-M. 2008. Is my antibody-staining specific? How to deal with pitfalls of immunohistochemistry. Eur J Neurosci 28:2365-2370.

Furness DN, Dehnes Y, Akhtar AQ, Rossi DJ, Hamann M, Grutle NJ, Gundersen V, Holmseth S, Lehre KP, Ullensvang K, Wojewodzic M, Zhou Y, Attwell D, Danbolt NC. 2008. A quantitative assessment of glutamate uptake into hippocampal synaptic terminals and astrocytes: new insights into a neuronal role for excitatory amino acid transporter 2 (EAAT2). Neuroscience 157:80-94.

Hoffman EM, Schechter R, Miller KE. 2010. Fixative composition alters distributions of immunoreactivity for glutaminase and two markers of nociceptive neurons, Nav1.8 and TRPV1, in the rat dorsal root ganglion. J Histochem Cytochem 58:329-344.

Holmseth S, Dehnes Y, Bjørnsen LP, Boulland JL, Furness DN, Bergles D, Danbolt NC. 2005. Specificity of antibodies: unexpected cross reactivity of antibodies directed against the EAAT3 (EAAC) glutamate transporter. Neuroscience 136:649-660.

Holmseth S, Lehre KP, Danbolt NC. 2006. Specificity controls for immunocytochemistry. Anat Embryol (Berl) 211:257- 266.

Holmseth S, Scott HA, Real K, Lehre KP, Leergaard

TB, Bjaalie JG, Danbolt NC. 2009. The concentrations and distributions of three C-terminal variants of the GLT1 (EAAT2; slc1a2) glutamate transporter protein in rat brain tissue suggest differential regulation. Neuroscience 162:1055-1071.

James JA, Mcclain MT, Koelsch G, Williams DG, Harley JB. 1999. Side- chain specificities and molecular modelling of peptide determinants for two anti-Sm B/B' autoantibodies. J Autoimmun 12:43-49.

Josephsen K, Smith CE, Nanci A. 1999. Selective but nonspecific immunolabeling of enamel protein-associated compartments by a monoclonal antibody against vimentin. J Histochem Cytochem 47:1237-1245.

Kalyuzhny AE. 2009. The dark side of the immunohistochemical moon: industry. J Histochem Cytochem 57:1099-1101.

Lehre AC, Rowley NM, Zhou Y, Holmseth S, Guo C, Holen T, Hua R, Laake P, Olofsson AM, Poblete-Naredo I, Rusakov DA, Madsen KK, Clausen RP, Schousboe A, White HS, Danbolt NC. 2011. Deletion of the betaine-GABA transporter (BGT1; slc6a12) gene does not affect seizure thresholds of adult mice. Epilepsy Res 95:70-81.

Lehre KP, Danbolt NC. 1998. The number of glutamate transporter subtype molecules at glutamatergic synapses: chemical and stereological quantification in young adult rat brain. J Neurosci 18:8751-8757.

Lehre KP, Levy LM, Ottersen OP, Storm-Mathisen J, Danbolt NC. 1995. Differential expression of two glial glutamate transporters in the rat brain: quantitative and immunocytochemical observations. J Neurosci 15:1835- 1853.

Liu QR, López-Corcuera B, Mandiyan S, Nelson H, Nelson N. 1993. Molecular characterization of 4 pharmacologically distinct alpha-aminobutyric acid transporters in mouse brain. J Biol Chem 268:2106-2112.

Lorincz A, Nusser Z. 2008. Specificity of immunoreactions: the importance of testing specificity in each method. J Neurosci 28:9083-9086.

Lowry OH, Roberts NR, Leiner KY, Wu ML, Farr AL, Albers RW. 1954. The quantitative histochemistry of brain, III. ammon's horn. J Biol Chem 207:39-49.

Ottersen OP. 1987. Postembedding light- and electron microscopic immunocytochemistry of amino acids: description of a new model system allowing identical conditions for specificity testing and tissue processing. Exp Brain Res 69:167-174.

Paavilainen L, Edvinsson A, Asplund A, Hober S, Kampf C, Ponten F, Wester K. 2010. The impact of tissue fixatives on morphology and antibody-based protein profiling in tissues and cells. J Histochem Cytochem 58:237-246.

Peghini P, Janzen J, Stoffel W. 1997. Glutamate transporter EAAC-1- deficient mice develop dicarboxylic aminoaciduria and behavioral abnormalities but no neurodegeneration. EMBO J 16:3822-3832.

Pines G, Danbolt NC, Bjørås M, Zhang Y, Bendahan A, Eide L, Koepsell H, Storm-Mathisen J, Seeberg E, Kanner BI. 1992. Cloning and expression of a rat brain L- glutamate transporter. Nature 360:464-467.

Pool CW, Buijs RM (1988) Antigen identity in immunocytochemistry. In: Molecular Neuroanatomy. Van LB, eds. Elsevier Science Publishers B. V. Biomedical Division. . .

Pradidarcheep W, Labruyere WT, Dabhoiwala NF, Lamers WH. 2008. Lack of specificity of commercially available antisera: better specifications needed. J Histochem Cytochem

56:1099-1111.

Rhodes KJ, Trimmer JS. 2006. Antibodies as valuable neuroscience research tools versus reagents of mass distraction. J Neurosci 26:8017-8020.

Saper CB. 2005. An open letter to our readers on the use of antibodies. J Comp Neurol 493:477- 478.

Saper CB. 2009. A guide to the perplexed on the specificity of antibodies. J Histochem Cytochem 57:1-5.

Saper CB, Sawchenko PE. 2003. Magic peptides, magic antibodies: guidelines for appropriate controls for immunohistochemistry. J Comp Neurol 465:161-163.

Shelanski ML, Gaskin F, Cantor CR. 1973. Microtubule assembly in the absence of added nucleotides. Proc Natl Acad Sci U S A 70:765-768.

Sibille P, Ternynck T, Nato F, Buttin G, Strosberg D, Avrameas A. 1997. Mimotopes of polyreactive anti-DNA antibodies identified using phage-display peptide libraries. Eur J Immunol 27:1221-1228.

Swaab DF, Pool CW, Van Leeuwen FW. 1977. Can specificity ever be proved in immunocytochemical staining. J Histochem Cytochem 25:388-391.

Tanaka K, Watase K, Manabe T, Yamada K, Watanabe M, Takahashi K, Iwama H, Nishikawa T, Ichihara N, Hori S, Takimoto M, Wada K. 1997. Epilepsy and exacerbation of brain injury in mice lacking the glutamate transporter GLT- 1. Science 276:1699-1702.

Webster JD, Miller MA, Dusold D, Ramos-Vara J. 2009. Effects of prolonged formalin fixation on diagnostic immunohistochemistry in domestic animals. J Histochem Cytochem 57:753-761.

Willingham MC. 1999. Conditional epitopes. is your antibody always specific?. J Histochem Cytochem 47:1233- 1236.

Xie R, Chung J-Y, Ylaya K, Williams RL, Guerrero N, Nakatsuka N, Badie C, Hewitt SM. 2011. Factors influencing the degradation of archival formalin-fixed paraffin-embedded tissue sections. J Histochem Cytochem 59:356-365.

IV

Title: Low density of EAAC1 (EAAT3; slc1a1) glutamate transporters suggests involvement in neuronal metabolism rather than in rapid control of synaptically released glutamate

Authors: Holmseth S[1], Dehnes Y[1], Huang, YH[3], Follin-Arbelet VV[1], Grutle NJ[1], Mylonakou NM[1], Plachez C[4], Zhou Y[1], Furness DN[2], Bergles DE[3], Lehre KP[1]* and Danbolt NC[1]*
Affiliations of all authors:
[1] Centre of Molecular Biology and Neuroscience, Department of Anatomy, Institute of Basic Medical Sciences, University of Oslo, P.O.Box 1105 Blindern, N 0317 Oslo, Norway.
[2] Institute for Science and Technology in Medicine, Keele University, Keele, Staffs, ST5 5BG, England
[3] Department of Neuroscience, Johns Hopkins University School of Medicine, WBSB 813, 725 North Wolfe St., Baltimore, MD 21205, USA
[4] Department of Anatomy and Neurobiology, University of Maryland, School of Medicine, Baltimore, MD

Abbreviated title: The concentration of EAAC1

Number of text pages, figures and tables:
nn text pages, 15 figures and 3 tables

Number of words in the
- abstract: 250 (max 250)
- introduction: 494 (max 500)
- discussion: 1491 (max 1500)

Address of corresponding author:
Centre of Molecular Biology and Neuroscience, Department of Anatomy
Institute of Basic Medical Sciences, University of Oslo, P.O.Box 1105 Blindern, N-0317 Oslo, NORWAY. Fax: (+47) 22 85 12 78, E-mail: n.c.danbolt@medisin.uio.no.

Acknowledgements: This work was supported by the Norwegian Research Council (fellowships to YD, and KPL). NCD acknowledges the economic support of the Norwegian Advanced Research Program (Toppforskningsprogrammet covering SH's salary), FUGE II (183727-S10) and a private donation which financed the synthesis of the C510 and the C491 peptides. We are especially grateful to Rolf Seljelid without whose encouragement and support the project would not have been possible. Correspondence should be addressed to: Knut Petter Lehre or Niels Chr. Danbolt, Department of Anatomy, the Institute of Basic Medical Sciences, University of Oslo, P.O.Box 1105 Blindern, N-0317 Oslo, Norway. Fax: (+47) 22 85 12 78. E-mail: k.p.d.lehre@medisin.uio.no, n.c.danbolt@medisin.uio.no.

Keywords: Glutamate uptake, Neurotransmitter transport, Antibodies, Quantitative immunoblotting, Membrane protein purification

ABBREVIATIONS

The abbreviations used are: BSA, bovine serum albumin; CHAPS, 3-[(3-cholamido propyl)dimethylammonio]-1-propanesulphonate; EAAC1, glutamate transporter subtype 3; EAAT, Excitatory amino acid transporter (= glutamate transporter); EDTA, sodium ethylenediamine tetraacetate; GABA, aminobutyric acid; GFAP; glial fibrillary acidic protein; GLAST, glutamate transporter subtype 1; GLT1, glutamate transporter subtype 2; HEPES, 4-(2-hydroxyethyl)-1- piperazineethanesulfonic acid; MBP, myelin basic protein; MK-801, dizocilpine; NBQX, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline; PMSF, phenylmethanesulfonyl fluoride; Rb, rabbit; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Sh, sheep; TBOA; DL-*threo*-β-benzyloxyaspartate; TBST, Tris-buffered saline with 0.1% Triton X-100; TTX, tetrodotoxin; SR95531, 6-imino-3-(4-methoxyphenyl)-1(6*H*)-pyridazinebutanoic acid HBr.

ABSTRACT

GLAST and GLT1 are the most abundant glutamate transporters in the mammalian brain, and are essential for harnessing glutamate. The roles of the other glutamate transporters are poorly understood. In particular, studies of EAAC1, the predominant neuronal transporter, have arrived at different conclusions in part because the density of EAAC1 is unknown and because of variable specificity of available antibodies. We isolated EAAC1 protein, and used the pure protein in known amounts as standards during immunoblotting to determine the concentration of EAAC1 in the hippocampus of young adult Wistar rats. The concentration was about 0.013 mg/gm tissue (about 130 molecules μm^{-3}) and thus 100 times lower than that of GLT1. The EAAC1 concentration increased from E18 to P14 and then decreased by a factor of three by adulthood. Using EAAC1 knockout mice as negative controls to establish antibody specificity, we show that EAAC1 is present in somata and dendrites of all hippocampal neurons and not in axon-terminals, astrocytes or oligodendrocytes. If all EAAC1 transporters were trafficked to the membrane, the average density would be approximately 90 μm^{-2} assuming a surface density of dendrites and spines of 1.5 $\mu m^2/\mu m^3$. The effective number is probably lower because most of EAAC1 is intracellular. Photolysis of MNI-D-aspartate failed to elicit EAAC1-mediated transporter currents in CA1 pyramidal neurons. D-aspartate uptake was not detected electron microscopically in spines. This and the low glutamate uptake activity in the GLT1 knockout mice suggest that EAAC1 is involved in neuronal metabolism, rather than rapid clearance of synaptically released glutamate.

INTRODUCTION

The extracellular levels of the neurotransmitter glutamate have to be tightly controlled to ensure high fidelity in synaptic transmission and to prevent excitotoxicity. This is accomplished by cellular uptake (Danbolt, 2001). The most important glutamate transporter subtypes are GLAST (EAAT1) and GLT1 (EAAT2). The roles of the third subtype, EAAC1 (EAAT3, slc1a1), is still debated. Some data suggest that EAAC1 is primarily involved in metabolic processes. For instance, immunisolation of transport activity (Haugeto et al., 1996), deletion of the GLT1 (slc1a2) gene (Tanaka et al., 1997; Bergles and Jahr, 1997; Bergles and Jahr, 1998; Sun et al., 2011), and the mild phenotype of EAAC1-deficient mice (Peghini et al., 1997) indicate that EAAC1-mediated glutamate uptake is negligible compared to that of GLT1. EAAC1-deficient mice suffer from dicarboxylic aminoaciduria and from premature ageing (Aoyama et al., 2006; Chen and Swanson, 2003; Berman et al., 2011), but do not exhibit overt CNS phenotypes suggestive of impaired glutamatergic or GABAergic signaling. Observations of humans with defective EAAC1 are in line with this view (Bailey et al., 2011). On the other hand, antisense knockdown indicates that the EAAC1 subtype accounts for about 40 % of the glutamate uptake activity in the hippocampus (Rothstein et al., 1996), and high resolution immunolabeling studies (He et al., 2000; He et al., 2001) have concluded that EAAC1 is present in dendritic shafts and in spines surrounding active zones as well as in terminals. At hippocampal synapses in EAAC1-deficient mice, glutamate transporter currents in astrocytes decay more rapidly, suggesting that the predominant action of EAAC1 is to buffer, rather than rapidly transport glutamate (Scimemi et al., 2009). This buffering may increase the probability of glutamate capture by GLAST and GLT1 that are present at high densities in astrocytic processes (Lehre and Danbolt, 1998). By this mechanism, EAAC1 could limit activation of perisynaptic NMDA receptors and increase the threshold for induction of long term potentiation (Scimemi et al., 2009). Further, functional studies suggest that EAAC1 is also present in GABAergic nerve terminals, where it may help maintain GABA levels by providing glutamate for GABA synthesis (Sepkuty et al., 2002; Mathews and Diamond, 2003; Stafford et al., 2010). Several of these studies are of excellent quality and it is hard to judge who is right. This is further complicated by the fact that different model systems have different limitations and that EAAC1 can be dramatically upregulated (Ross et al., 2011). Thus, the role of EAAC1 may differ between preparations. To resolve this conundrum, we have quantified the amounts of EAAC1 protein relative to GLT1 protein, and we show that EAAC1 is approximately 100-fold less abundant than GLT1 in the young adult rat hippocampus. Further, we validated the specificity of EAAC1 antibodies using tissue from EAAC1-deficient mice, and determined the EAAC1 distribution immunocytochemically. We find EAAC1 in dendrites of all cells identified as neurons, but not in axons, terminals, oligodendrocytes or astrocytes. The data support the hypothesis that EAAC1 plays a role in neuronal metabolism rather than neurotransmission.

MATERIALS AND METHODS

Materials

Sodium dodecyl sulfate (SDS) of high purity (>99 % C12 alkyl sulfate), bis(sulfosuccinimidyl)suberate, SuperSignal West Dura® were from Pierce (Rockford, IL, USA) and electrophoresis equipment were from Hoefer Scientific Instruments (San

Francisco, CA, USA). N,N'-methylene-bisacrylamide, acrylamide, ammonium persulfate, TEMED and alkaline phosphatase substrates (nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate) were from Promega (Madison, WI, USA). Protein A-Sepharose Fast Flow and Sephadex G-50 fine were from Pharmacia (Uppsala, Sweden). Molecular mass markers for SDS-polyacrylamide gel electrophoresis (SDS-PAGE), ECL-films and Nitrocellulose sheets (0.22 µm pores, 100 % nitrocellulose) were from Amersham (Buckinghamshire, UK). Paraformaldehyde and glutaraldehyde EM grade were from TAAB (Reading, UK). Lowicryl HM20 was from Electron Microscopy Sciences (Fort Washington, PA, USA). Bovine serum albumin (BSA), 3-[(3-cholamido-propyl) dimethylammonio]-1-propanesulphonate (CHAPS), dimethyl suberimidate, dithiotreitol, sodium ethylenediamine tetraacetate (EDTA), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), human serum albumin, phenylmethanesulfonyl fluoride (PMSF), Trizma base, Trisma-HCl, Triton X-100 and wheat germ agglutinin were obtained from Sigma (St. Louis, MO, USA). Wheat germ agglutinin was immobilized on agarose as described previously (Danbolt et al., 1992). Pure EAAT2 glutamate transporter protein was from the same batches as previously produced (Lehre and Danbolt, 1998).

Antibodies

Anti-peptide antibodies against glutamate transporters were from the same batches as described previously. The batch numbers are given below together with reference: The anti-GLAST antibodies were the rabbit anti-A522 antibody (Ab#141; Lehre et al., 1995) and a mouse monoclonal anti-GLAST antibody (lot 124102; NCL-EAAT1; Novocastra Laboratories, Newcastle, UK; Banner et al., 2002). Both of these were directed to a peptide (PYQLIAQDNEPEKPVADSET-*amide*) representing residues 522-541 of rat GLAST (Storck et al., 1992). The anti-GLT1 antibodies were the monoclonal 9C4 antibody (Ab#531; Levy et al., 1993), the anti-B12 (Ab#360; Holmseth et al., 2005) to residues 12-26 of rat GLT1 (KQVEVRMHDSHLSSE-*amide;* Pines et al., 1992) and a monoclonal antibody NCL-EAAT2 (lot 118003; Novocastra Laboratories, Newcastle, UK). Three synthetic peptides representing parts of EAAC1 (Kanai and Hediger, 1992; Bjørås et al., 1996) were used to produce the anti-EAAC1 antibodies used in the present report: C479 (IVNPFALEPTILDNEDSDTK-*amide*), C491 (CLDNEDSDTKKSYVNGGFSVDKSDTISFTQTSQF-*free acid*) and C510 (VDKSDTISFTQTSQF-*free acid*). The rabbit antibodies to the C479 peptide (anti-C479; Ab#359) cross-reacted with tubulin and were therefore absorbed against immobilized tubulin before being used in the present study (Holmseth et al., 2005). The anti-C491 antibodies were from several different batches (Ab#237, Ab#236, Ab#371 and Ab#555) and have also been described (Holmseth, Dehnes, et al. 2006 #14000}). The anti-C510 antibodies were both from rabbit (Ab#126; Haugeto et al., 1996) and from sheep (Ab#340; Holmseth, Dehnes, et al. 2006 #14000}). The latter batch was further purified by passing it through a column with aldehyde treated bacterial proteins to remove unwanted antibodies (Danbolt et al., 1998). The antibodies that did not stick to the column were highly specific (Ab#565; Holmseth et al., 2011). Anti-glutamine synthetase (MAB 302) was from Chemicon (Temecula, CA, USA), Anti-NG2 (sc-20162) was from Santa-Cruz Biotechnology (Santa Cruz, CA, USA), anti-myelin basic protein (MBP) and anti-CNPase were Sternberger Monoclonals (Lutherville, MD, USA). Anti-VGLUT1 (clone 317D5) was from SYSY (Goettingen, Germany). Anti-parvalbumin (P3088, lot 30K4824), anti-glial fibrillary acid protein (G3893, lot 082K4834), anti-glutamin synthetase (G2781, lot 061K4811), anti-synaptophysin (S5768), anti-GAD

(G1166, clone GAD-6), alkaline phosphatase- and peroxidase-conjugated monoclonal antibodies to mouse, rabbit and sheep IgG (A5187, A9452, A2179, A2556, A9044 and A1949) were from Sigma-Aldrich (St. Louis, MO, USA). Biotinylated anti-rabbit, anti-sheep and anti-mouse immunoglobulins, streptavidin-biotinylated horseradish peroxidase complex, and colloidal gold labeled anti-rabbit and anti-mouse immunoglubulins were from Amersham (Buckinghamshire, UK). Alexa fluor goat anti-rabbit 555, goat anti-mouse 488 and donkey anti-sheep 555 were from Molecular probes (Eugene, OR, USA). Other reagents were obtained from Fluka (Buchs, Switzerland). The fluorescein *Lotus tetragonolobus* lectin (FL-1321, lot W0909) was from Vector Laboratories (Burlingame, CA, USA).

Animals

All animal experimentation was carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80 23) revised 1996 and the European Communities Council Directive of 24 November 1986 (86/609/EEC). Formal approval to conduct the experiments described was obtained from the animal subjects review board of our institutions. Care was taken to avoid suffering and minimize the number of animals used. Adult male Wistar rats (9-12 weeks old as indicated) and C57Black/6 (3 weeks old) obtained from B&K Universal (Sollentuna, Sweden) were kept in the animal facility at the Institute of Basic Medical Sciences. Brain tissue for immunocytochemistry was obtained from rats that had been killed by injection of pentobarbital and fixed by cardiac perfusion as described (Danbolt et al., 1998). The generation of mice lacking EAAC1 and their genotyping, have been described in detail previously (Peghini et al., 1997). Experiments were carried out on EAAC1 wild-type and knockout mice (3-4 weeks old). Animals used for electrophysiology recordings were 12-17 day-old Sprague-Dawley rats or mice.

Electrophoresis and blotting

SDS-PAGE was performed as described before (Lehre et al., 1995) with separating gels consisting of 7.5 or 10 % acrylamide, or with 4-20 % gradient gels. The molecular mass markers were used in non-reduced form. After electrophoresis the proteins were either silver stained or electroblotted onto nitrocellulose membranes. Because the purpose of the immunoblotting was to maximize the probability of detecting possible unwanted immunoreactivity towards non-EAAC1-proteins, the samples were prepared by homogenizing whole tissue directly in SDS so that the blots would contain as many of the tissue antigens as possible (for discussion of antibody specificity and testing see Holmseth et al., 2005).

Qualitative immunolabeling of blots (Fig. 1) was done using alkaline phosphatase conjugated secondary antibodies exactly as described previously (Lehre et al., 1995). This detection system has low contrast and is therefore suitable for detection of cross-reactivity. As shown (Fig. 1A-C), the antibodies used in this study labeled one weak, but relatively broad fuzzy band at around 70 kDa on immunoblots of fresh tissue from brain and kidney directly solubilized in SDS. Some weak bands representing non-EAAC1 proteins were also observed (e.g. Fig. 1A, strip 1), but the band representing EAAC1 was absent on blots prepared from tissue from EAAC1-deficient mice (Fig. 1D). This implied that the immunolabeling of this band could be used as a measure of the amount of EAAC1.

Immunoaffinity purification of EAAC1 from rat kidney

Antibodies (0.5 mg anti-C491; Ab#236 or Ab#555) were immobilized on protein A-Sepharose Fast Flow (0.5 ml) and covalently linked using dimethyl suberimidate as described previously (Danbolt et al., 1992). EAAC1 protein was purified three times (Table 1) using the following procedure: 20 kidneys (approximately 3 g protein) were freshly dissected from Wistar rats and homogenized in 10 volumes of ice-cold hypotone buffer (5 mM EDTA, 1 mM PMSF) using a Polytron7 PT1200 homogenizer followed by treatment in a Dounce glass-glass homogenizer. The mixture was centrifuged (20 min, 39000 x g, 4 ^{o}C, Beckman JA20 rotor). The pellet was solubilized either in 10 volumes solubilization buffer (0.5 % lithium dodecyl sulfate, 45 mM Li-HEPES pH 7.5, 90 mM LiCl, 4.5 mM EDTA and 0.9 mM PMSF) or in 2 % cholate (Lehre and Danbolt, 1998) as indicated (Table 1). The mixture was sonicated (30 s, on ice, with a dr.Hilscher type UP 50H® sonicator) to reduce viscosity by breaking up DNA and then incubated (5 min on ice). When lithium dodecyl sulfate had been used, then Triton X-100 was added to a final concentration of 25-30 mg/ml in order to make the solution "antibody friendly". The resultant mixture was incubated (5 min on ice), and centrifuged (39,000 x *g*, 20 min, 4°C, Beckman JA20 rotor). The supernatant was incubated end-over-end (90 – 270 min, 4°C) with the immobilized antibodies (see above). The antibody beads were washed (3 x 6 min, 4°C) with buffer (0.3 M NaCl, 20 mM CHAPS, 40 mM sodium phosphate buffer pH 7.4). Bound proteins were eluted (2 x 5 min, 4°C) with low-pH buffer (0.15 M NaCl, 20 mM CHAPS, 0.32 M glycine-HCl, pH 2.0). The eluate was immediately neutralized by adding 1/10 volume 2 M Tris-HCl (pH 9). EDTA and PMSF were added to final concentrations of 5 and 1 mM, respectively. The eluate was then passed through a DEAE-cellulose column or a wheat germ agglutinin-agarose column as indicated (Table 1). The wheat germ agglutinin-column was washed with 2 volumes washing buffer (20 mM CHAPS, 0.5 M NaCl and 20 mM sodium phosphate buffer pH 7.4) and bound proteins were eluted with NAG-buffer (0.200 mM N-acetylglucosamine, 20 mM CHAPS, 0.5 M NaCl and 20 mM sodium phosphate buffer pH 7.4). Aliquots of the eluate destined for SDS-PAGE were mixed with SDS sample buffer containing 50 mM dithiothreitol and frozen. SDS was added to stop the irreversible aggregation of transporters that occurs in mild detergents (for a discussion of SDS-insoluble multimers of glutamate transporters see section 7.4.2 in Danbolt, 2001)

Using this procedure highly purified rat kidney EAAC1 protein (Fig. 2, lanes 5-8) was obtained. There was no detectable leak of IgG from the affinity column. The IgG heavy chains give rise to a band just below that of EAAC1 (not shown). However, SDS-insoluble higher molecular mass aggregates formed during the purification procedure until the samples could be stabilized by the addition of SDS. This complicated protein measurement because non-aggregated and aggregated versions of the protein have different molecular masses and thereby have different blot transfer efficacies. Because only the monomer is seen on blots made from extracts prepared by direct solubilization of fresh tissue in SDS (Fig. 3A), only the monomer was useful as standard for immunoblotting. The concentrations of the monomer in the purified preparations of EAAC1 (Table 1) were determined densitometrically on silver stained polyacrylamide gels (Danbolt et al., 1990) using purified EAAT2 as a standard (Fig. 2). EAAT2-protein was chosen because this is a closely related protein which thereby presumably reacts similarly with the silver stain, because it was available in sufficient quantities to allow precise determination of its concentrations by a number of protein assays (including both the Lowry and the BCA assays) (Lehre and Danbolt, 1998), and because we have been able to produce this protein in non-aggregated form as shown (Fig. 2). The stained

gels were scanned using translumination on an Epson Expression 1680 Pro7 scanner and the density of the bands was determined both with ImageJ (National Institutes of Health, USA) and with ImageMaster TotalLab (Amersham). The two programs gave the same results. Because the immunoaffinity isolation method is expensive with regard to antibodies, the antigen was always added in excess to ensure saturation of the antibodies. Under these conditions, 0.5 ml of gel containing 0.5 mg of immobilized antibodies gave 90-130 µg EAAC1 protein and 12-20 µg remained after the final purification on the wheat germ agglutinin-column.

Immunolabeling of blots for quantitative measurements
Quantitative measurements were made either using ^{125}I-protein A exactly as described (Beckstrøm et al., 1999) in combination with anti-C491 (Ab#237; 0.1 - 3 µg/ml) or using chemoluminescence (Supersignal West Dura™ from Pierce, Rockford, IL, USA) as described (Holmseth et al., 2009) in combination with anti-C491 (Ab#371, 0.125 µg/ml). The immunoreactivities per unit protein in whole tissue homogenates of hippocampus, cerebellum and kidney were compared with that of three purified preparations of EAAC1 (Table 1). The hippocampal and cerebellar homogenates contained all tissue components because SDS dissolves brain tissue completely (a clear solution is obtained). The kidney, however, contains water and SDS insoluble components (connective tissue). Therefore the kidney homogenates refer only to the components that could be solubilzed. When stated (Table 2) hippocampus was first homogenized in water and subjected to high speed centrifugation (39,000 x *g*, 20 min, 4°C, Beckman JA20 rotor). The pellet was dissolved in SDS as above and referred to as 'Membranes' in Table 2. To determine the regional differences in EAAC1-levels (Fig. 3) whole tissue homogenates were made from cerebellum, neocortex, olfactory bulb, striatum, thalamus/hypothalamus, pons and mesencephalon. The immunoreactivities of these homogenates were compared to that of hippocampus using the chemiluminescence based method only. The samples were run in triplicates and compared to hippocampus (Fig. 3A).

Immunocytochemistry
Perfusion fixation of animals, Vibratome sectioning, and immunoperoxidase staining of Vibratome sections, and pre-embedding electron microscopy were done as described before (Danbolt et al., 1998). Fluorescence labeling and confocal microscopy were done as previously (Holmseth et al., 2005), while post-embedding immunogold immunocytochemistry was done according to Furness et al. (2008). The sections were 40 µm thick.

Estimation of neuronal surface density
This was done in the stratum radiatum of hippocampus (subfield CA1) about 4 mm from the temporal pole using the same material as we have previously used for measuring the glial surface density (Lehre and Danbolt, 1998). Tissue components were identified according to Peters et al. (1991) and Palay and Chan-Palay (1974). Photographs from serial sections were obtained because it is not possible to identify all the cellular processes in a single picture, and because this study required identification of all cellular processes in order to obtain a measure for the total cell surface. The lengths of all identified dendritic (including spines) and astroglial cell membranes, and the area of the analyzed images (104 µm^2), were measured by a computer program from Science Linker AS (Oslo, Norway). Surface areas of dendrites

and astroglia were calculated by multiplying the membrane lengths per image area by 4/pi (Weibel, 1979).

Electrophysiology

Slice Preparation: Hippocampal slices were prepared from 12-17 day-old Sprague-Dawley rats or mice, in accordance with a protocol approved by the Animal Care and Use Committee at Johns Hopkins University. Animals were anesthetized with halothane and decapitated, the hippocampi were dissected free, mounted in agar blocks, cut in 400 □m thick transverse sections using a vibratome (VT1000S, Leica), in oxygenated ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl 119, KCl 2.5, CaCl2 2.5, MgCl2 1.3, NaH2PO4 1, NaHCO3 26.2 and D Glucose 11. Slices were allowed to recover on a gauze net submerged in ACSF at 37° C for 30 minutes and kept at room temperature thereafter. For cerebellar slices, 250 □m thick parasagittal sections were prepared.

Whole Cell Recording: Individual brain slices were placed in a chamber mounted on an upright microscope (Zeiss, Axioskop FS2) and continuously superfused with oxygenated ACSF. Individual cells (astrocytes, CA1 pyramidal neurons, or Purkinje neurons) were visualized through a 40x water immersion objective (Olympus LUMPlanFL, NA = 0.8) using infrared-Nomarski optics and a CCD camera (Sony XC-73). Recording electrodes were pulled from glass capillary tubing and had a combined resistance of 1.5-3.0 MΩ when filled with the internal solution. For astrocytes, the internal solution contained (in mM): KCH_3O_3S (KMeS) 120, EGTA 10, HEPES 20, $MgCl_2$ 1, Na_2ATP 2, NaGTP 0.2; the pH was 7.3. To record transporter associated anion currents from CA1 pyramidal neurons and Purkinje neurons, the internal solution contained (in mM): $CsNO_3$ 100, TEA-Cl 20, EGTA 10, HEPES 20, $MgCl_2$ 1, QX-314 1, Na2ATP 2, NaGTP 0.2; the pH was 7.3. With these solutions the series resistance during recordings was <10 MΩ, and was left uncompensated. Unless stated otherwise, holding potentials have not been corrected for the junction potential. Whole-cell currents were amplified using an Axon Multiclamp 700A (Axon Instruments), filtered at 2-5 kHz and sampled at 10-20 kHz.

Solution Application: Caged compounds were dissolved in HEPES buffered saline (HEPES ACSF) containing (in mM): NaCl 137, KCl 2.5, $CaCl_2$ 2.5, $MgCl_2$ 1.3, HEPES 20; the pH was 7.3. Solutions containing caged compounds were applied to the slice using a wide bore (tip diameter 50-100 □m) pipette connected to a manifold fed by four 10 ml reservoirs. Solutions were switched by alternately opening and closing valves attached to each reservoir. Antagonists were used to block voltage-gated Na+ channels (tetrodotoxin; TTX, 1 □M), AMPA/kainate receptors (2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide disodium salt; NBQX, 10 □M), NMDA receptors ((RS)-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid; R,S-CPP, 10 □M; and (5R,10S)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclo-hepten-5,10-imine hydrogen maleate; MK-801, 50 □M), and GABAA receptors (6-imino-3-(4-methoxyphenyl)-1(6H)-pyridazinebutanoic acid dihydrobromide; SR-95531, 5 □M). In some experiments, group I mGluRs were blocked with LY367385 (100 □M). Glutamate transporters were inhibited using DL-threo-□-benzyloxyaspartic acid (TBOA, 100-200 □M).

Photolysis: UV light (333.6-363.8 nm) was delivered to the sample by coupling the output of an argon ion laser (Stabilite 2017-AR, Spectra-Physics) to a multi-mode quartz fiber optic cable (Oz Optics Ltd.). The output of the fiber optic was collimated using a quartz lens, projected through the fluorescence port of a Zeiss Axioskop FS2 microscope, and focused to a ~50 □m spot using a 40x water immersion objective (Olympus LUMPlanFl). MNI-D-

aspartate was applied to cells locally through a wide bore pipette, and photolysis achieved by brief (~1 ms) exposure to UV light, as described (Huang et al., 2005).

Preparation of hippocampal slices from adult rat brains
Hippocampal slices were prepared and incubated with D-aspartate essentially as described previously (Furness et al., 2008). Briefly, fresh rat hippocampal slices were incubated in Krebs' phosphate solution (140 mM NaCl, 5 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 10 mM glucose and 15 mM sodium phosphate buffer, pH 7.4) with 1 - 50 μM D-aspartate. Negative controls were treated as above, but incubated either in the absence of D-aspartate or with the addition of 1 mM L-glutamate. Following incubation, slices were fixed using 1 % formaldehyde and 2.5 % glutaraldehyde in 0.1 M sodium phosphate buffer, embedded by freeze substitution (Dehnes et al., 1998) and processed for immunogold transmission electron microscopy.

Reconstitution of glutamate transporters into proteoliposomes
This was done as previously described (Danbolt et al., 1990). Briefly, crude rat brain was homogenized in 10-20 volumes of ice-cold water with 5 mM EDTA and 1 mM PMSF. After centrifugation (39000 x g, 20 min, 4°C), the pellet was solubilized and centrifuged as above. The supernatant was mixed with a phospholipid cholate salt mixture, incubated on ice and gel filtered (Trotti et al., 1995) to remove detergent and sodium ions equilibrated with the desired internal medium.

RNA isolation, cDNA synthesis and TaqMan Assays
This was done as described previously (Lehre et al., 2011) Pieces of tissue (5-15 mg) were collected and immediately protected in RNAlater (Ambion/Applied Biosystems, TX, USA), before homogenization and RNA extraction using RNeasy® (Qiagen, Hilden, Germany). The RNA concentration was quantified by using NanoDrop UV spectrometry (NanoDrop Technologies, Wilmington, USA). Two μg RNA were converted into cDNA by using High Capacity cDNA Archive Kit (Applied Biosystems; Carlsbad, CA, USA). EAAC1 mRNA expression was measured using TaqMan probes (Mm00436590_m1) on a 7900HT Fast Real-Time PCR system (Applied Biosystems; Carlsba, CA, USA). The probe which was used to detect GAPDH (as control) was Mm03302249_g1.

RESULTS

The concentration of EAAC1 protein in young adult rat hippocampus and kidney
Antibodies to EAAC1 were produced (Fig. 1) and used to immunoisolate EAAC1 protein from rat kidney. Three highly purified preparations of EAAC1 protein were obtained (Fig. 2, lanes 5-8; Table 1). To circumvent the problem of partial aggregation (see Methods), the concentrations of the monomer in these preparations were determined densitometrically on silver stained gels by comparing with known amounts of pure GLT1 protein (Fig. 2, lanes 1-4). Then, immunoreactivity of the EAAC1 monomer in the three purified preparations (Table 1) was compared with that of the EAAC1 monomers in tissue extracts (Fig. 3a; Table 2) and found to correspond to 0.10 ± 0.011 mg, 0.16 ± 0.004 mg and 0.13 ± 0.013 mg EAAC1 monomer, respectively (mean ± SEM), per gram total protein. From this, we concluded that the concentration of EAAC1-protein in young adult rat hippocampus tissue is about 0.013 ± 0.02 mg per gram (tissue wet weight). For kidney whole-tissue extracts the corresponding

Detergent	Rat age	Antibody ID	Amount of pure EAAC1 protein	WGA	DEAE	Sample ID
Cholate	220-240 gm	Ab#237	88 µg	No	Yes	1374
SDS	9 w	Ab#555	12 µg	Yes	No	1357
SDS	9 w	Ab#555	20 µg	Yes	No	1358

Table 1. Isolation of EAAC1 protein from rat kidney : Rat kidneys were solubilized in cholate or SDS and EAAC1 isolated using immobilized anti-C491 antibodies (either Ab#237 or Ab#555). The bound EAAC1 protein was eluted at low pH, neutralized and further purified by chromatography on either a wheat germ agglutinin lectin (WGA) column or a diethylaminoethyl (DEAE) column as indicated. The age is given in weight (gm) or in weeks (w).

numbers were 0.014 ± 0.001 and 0.02 ± 0.002 mg per gram tissue (from two purified preparations, Table 1: #1357 and #1358). In the cerebellum, the amount was found to be lower, around 0.003 ± 0.0005 mg per gram cerebellar tissue (based on #1374). When the molecular mass of EAAC1 (58 kDa) is taken into account, it follows that the EAAC1 concentration in hippocampal neuropil is 1.3 x 10^{17} molecules per liter tissue or 0.22 µM, corresponding to about 130 EAAC1 molecules per cubic micrometer (Table 3). As there is approximately one nerve terminal per cubic micrometer in the rat stratum radium (Woolley and McEwen, 1992), it follows that there are on average about 130 EAAC1 transporters at each excitatory synapse in this region.

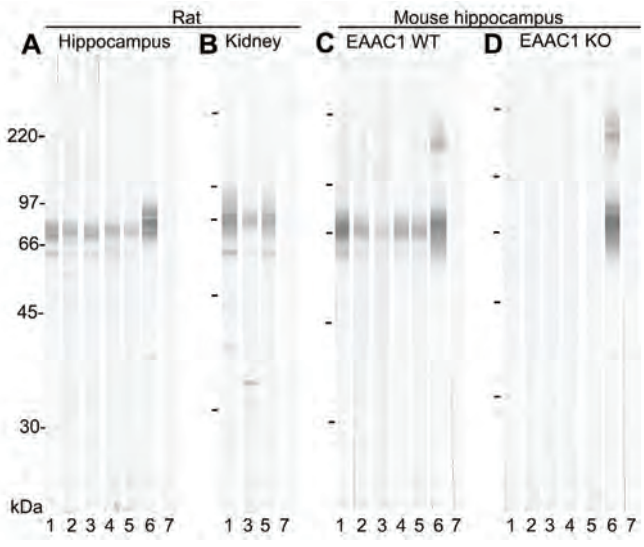


Figure 1. The specificity of the EAAC1 antibodies as demonstrated by immunoblotting of electrophoretically separated proteins from (A) rat hippocampus, (B) rat kidney, and (C) wild-type and (D) EAAC1 knockout mice hippocampi. The blot strips were incubated with: Lane 1, anti-C491 (Ab#371); lane 2, anti-C491 (Ab#237); lane 3, anti-C479 (Ab#359); lane 4, sheep anti-C510 (Ab#565); lane 5, anti-C510 (Ab#126); lane 6, anti-B12 (Ab#152), (positive control), and lane 7, no primary antibody (negative control). Each strip contained 16 µg of protein. The absence of labeling in EAAC1-knock out mice (Panel D, lane 1-5) attests the specificity of the antibodies. All antibodies were used at a concentration of 1 µg/ml except for anti-B12 which was used at 0.2 µg/ml.

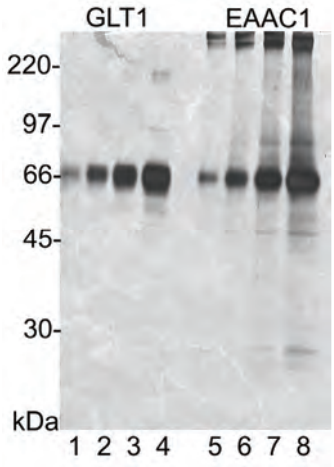


Figure 2. Determination of the concentration of monomeric EAAC1-protein in the purified preparations of EAAC1 protein. Highly purified GLT1 protein (50, 100, 200 and 350 ng in lanes 1-4, respectively) and a purified preparation of EAAC1 (5, 10, 20 and 35 µl in lanes 5-8, respectively) were subjected to SDS-PAGE and silver stained (Danbolt et al., 1990). The optical densities of the GLT1 bands were used as a standard to determine the protein concentration of the EAAC1 sample.

Glutamate uptake in GLT1-deficient mice

The above data suggest that EAAC1 is expressed at levels that are about 100 times lower than those of GLT1 (Table 3). This is in agreement with the low uptake activities observed in synaptosome preparations from GLT1 deficient mice (Tanaka et al., 1997). However, we have recently shown that synaptosome preparations mostly detect GLT1 catalyzed glutamate uptake into terminals (Furness et al., 2008). The possibility therefore existed that EAAC1 expressed in other compartments could have been underestimated. To obtain an estimate for the total uptake activity of all transporters in the tissue independently of whether they are present in cellular compartments that do not reseal after homogenization, we solubilized the tissue in cholate and reconstituted the transporters in artificial cell membranes (liposomes) containing internal potassium as we have done previously (Danbolt et al., 1990). These liposome preparations did not give any indications that EAAC1 is a major player because the glutamate uptake activity in liposomes prepared from GLT1 knockout tissue (forebrain) was very low (about 2 % of wild-type) compared to that of liposomes prepared from wild-type tissue (Fig. 4A). GABA uptake activity was used as a positive control, and was found to be similar in liposomes made from wild type and GLT1 knockout mice (Fig. 4B). The ionophore nigericin was used as a negative control because it compromises the transmembrane ion gradients. It abolished all uptake activity (of both glutamate and GABA). Further, immunoblots showed that GLT1-deficient mice have normal levels of EAAC1 (Fig. 4C). Although EAAT4 was readily detected in the cerebellum, detection of the tiny amounts of EAAT4 present in the forebrain requires other methods (see: Dehnes *et al.,* 1998).

Animal	Age	Region	Type	Detection system	mg EAAC1 per gram total protein
1	220 gm	Hippoc	Whole	^{125}I-Prot A	0.20
2	220 gm	Hippoc	Whole	^{125}I-Prot A	0.17
3	220 gm	Hippoc	Whole	^{125}I-Prot A	0.13
1	220 gm	Cerebel	Whole	^{125}I-Prot A	0.038
2	220 gm	Cerebel	Whole	^{125}I-Prot A	0.031
3	220 gm	Cerebel	Whole	^{125}I-Prot A	0.021
4	220 gm	Hippoc	Membr	^{125}I-Prot A	0.27*
5	220 gm	Hippoc	Membr	^{125}I-Prot A	0.25*
6	220 gm	Hippoc	Membr	^{125}I-Prot A	0.24*
7	18 w	Hippoc	Membr	^{125}I-Prot A	0.16*
8	8 w, f	Hippoc	Membr	^{125}I-Prot A	0.19*
9	8 w, f	Hippoc	Membr	^{125}I-Prot A	0.17*
10	14 w	Hippoc	Whole	Chemolum	0.08
11	14 w	Hippoc	Whole	Chemolum	0.11
12	14 w	Hippoc	Whole	Chemolum	0.08
13	9 w	Hippoc	Whole	Chemolum	0.11
14	9 w	Hippoc	Whole	Chemolum	0.13
15	9 w	Hippoc	Whole	Chemolum	0.16
16	9 w	Hippoc	Whole	Chemolum	0.17
17	9 w	Hippoc	Whole	Chemolum	0.16
18	9 w	Hippoc	Whole	Chemolum	0.15
19	9 w	Hippoc	Whole	Chemolum	0.17

Table 2. Quantification of EAAC1 protein in rat brain tissue: The amounts of EAAC1 protein in tissue from the hippocampus (Hippoc) and cerebellum (Cerebel) were measured by immunoblotting using purified preparations of EAAC1 protein (Table 1) as standards. The measurements of the levels in animals 1-9 are based on anti-C491 (Ab#237) and the first EAAC1 preparation (Table 1, Sample #1374) while the other measurements (animal 10-19) are based on anti-C491 (Ab#555) and the two other EAAC1 preparations. Bound antibodies were detected either with iodinated protein A or with chemoluminiscence (Chemolum) as indicated. Note that cerebellum contains less EAAC1 than hippocampus in agreement with figure 3. Also note that some tissue samples were homogenized in water to remove water soluble proteins before solubilization in SDS. These are indicated (Membr) in contrast to the rest which were solubilized directly (Whole). Water soluble proteins represent about 50 % of the total brain proteins, but do not contain any detectable amounts of EAAC1 (data not shown) the water insoluble fraction (Membr) are therefore 2-fold enriched with respect to EAAC1. The age is given in weight (gm) or in weeks (w). All animals were males except those indicated (f).

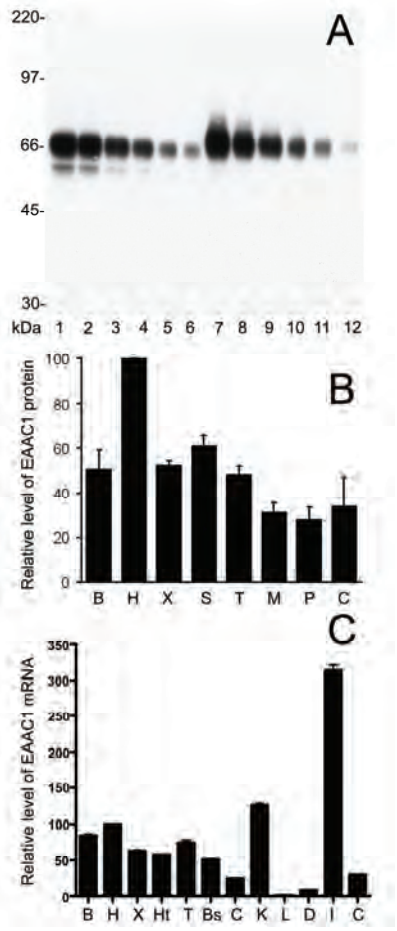


Figure 3. Quantification of EAAC1 protein and mRNA. **Panel A:** Immunoblot of hippocampus and purified EAAC1 (two different preparations were used as standards, only one of the preparations is shown here: 2.7, 1.35, 0.9, 0.45, 0.3, and 0.15 ng EAAC1 in lanes 7-12, respectively). The concentration of EAAC1 in hippocampus was measured by comparing the immunoreactivities of the tissue proteins (13.5, 9, 4.5, 3, 1.5, and 1 µg protein in lanes 1-6, respectively) with that of known amounts of the pure EAAC1 protein. **Panel B:** Quantitative distribution of EAAC1 in different regions of rat brain. Immunoblots with 2.5 µg protein/lane were reacted with 0.3 µg/ml anti-C491 (Ab#555). Bound antibodies were detected by chemiluminescence. The bands were located with exposure to X-ray film, and the different staining intensities on the film were analyzed with ImageJ (see Materials and methods). The results represent mean ± SEM of three sets of immunoblots (from three different Wistar rats aged 9 - 12 weeks). The values obtained from the region with highest immunoreactivity (hippocampus) were arbitrarily defined as 100 %, and the values from the other regions were expressed as percentage of this highest value. Regions: B, olfactory bulb; H, hippocampus; X, neocortex; S, corpus striatum; T, thalamus/hypothalamus; M, mesencephalon; P, pons; C: cerebellum. **Panel C:** The EAAC1 mRNA levels in mouse tissues (12 weeks old) including olfactory bulb(B), hippocampus (H), cortex (X), hypothalamus (Ht), thalamus (T), brain stem (Bs), cerebellum (C), kidney (K), liver (L), duodenum (D), ileum (I) and colon (C) were measured using TaqMan probe Mm00436590_m1 and expressed relative to the levels in hippocampus. Note that the highest levels of EAAC1 mRNA were in the intestine, kidney and hippocampus.

Regional distribution of EAAC1 protein and mRNA in the young adult rat

After having determined the concentration of EAAC1 protein in hippocampus, other brain regions were compared to hippocampus by immunoblotting (Fig. 3B). The concentrations in the striatum, neocortex, olfactory bulb, and thalamus/hypothalamus (in decreasing order) were approximately half of that in the hippocampus. The levels in the cerebellum, mesencephalon, and pons/medulla oblongata (in decreasing order) were at about one third to one quarter of the hippocampal level in agreement with the quantification (see above). These data are in reasonable agreement with the mRNA levels (Fig. 3C) and with the regional labeling intensity seen on tissue sections (Fig. 6) when the latter were processed in the presence of Triton X-100 to maximize penetration of the reagents (Lehre et al., 1995).

It was noted that the electrophoretic mobility of EAAC1 from striatum was slightly lower than that of EAAC1 from the other brain regions tested (data not shown) resembling the situation with cerebellar GLAST (EAAT1) which runs more slowly than that from the rest of the brain (Lehre et al., 1995).

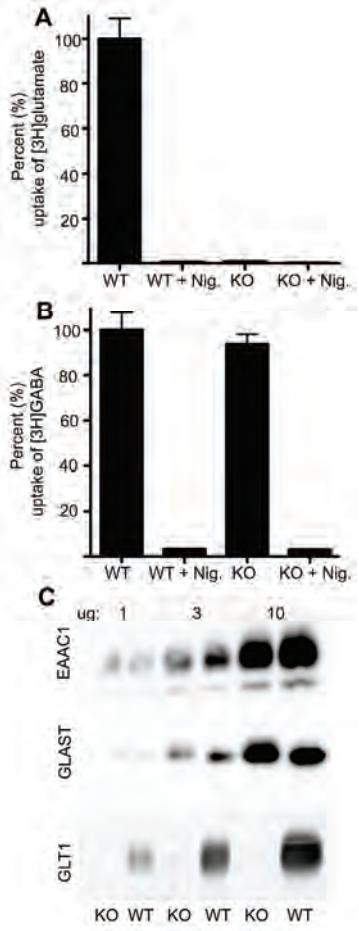


Figure 4. There is very little glutamate uptake activity remaining when GLT1 is knocked out. **Panel A:** L-[^{3}H]glutamate uptake in proteoliposomes prepared from forebrains from wild-type and GLT1 knockout mice. Note that the uptake in proteoliposomes prepared from GLT1 knockout tissue is negligible compared to those prepared from wild-type tissue. The uptake depends on the electrochemical gradients as illustrated by the fact that addition nigericin abolish all uptake activity. **Panel B:** GABA uptake activity in the same preparations of proteoliposomes. Note that the liposomes that were unable to take up L-[^{3}H]glutamate, take up [^{3}H]GABA as well as those prepared from wild-type tissue implying that the reconstitution process has been successful. **Panel C:** Immunoblots from forebrains of wild-type (WT) and GLT1 knockout (KO) mice show that GLT1 is absent while EAAC1 and GLAST is still present at normal levels. The amounts of protein in each lane were 1, 3 or 10 µg as indicated. The blots were developed with antibody anti-C479 (Ab#371; 1 µg/ml) for EAAC1, anti-A522 (Ab#314; 0.2 µg/ml) for GLAST and anti-B12 (Ab#355; 0.1 µg/ml) for GLT1.

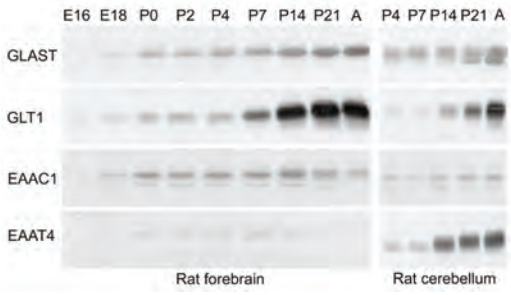
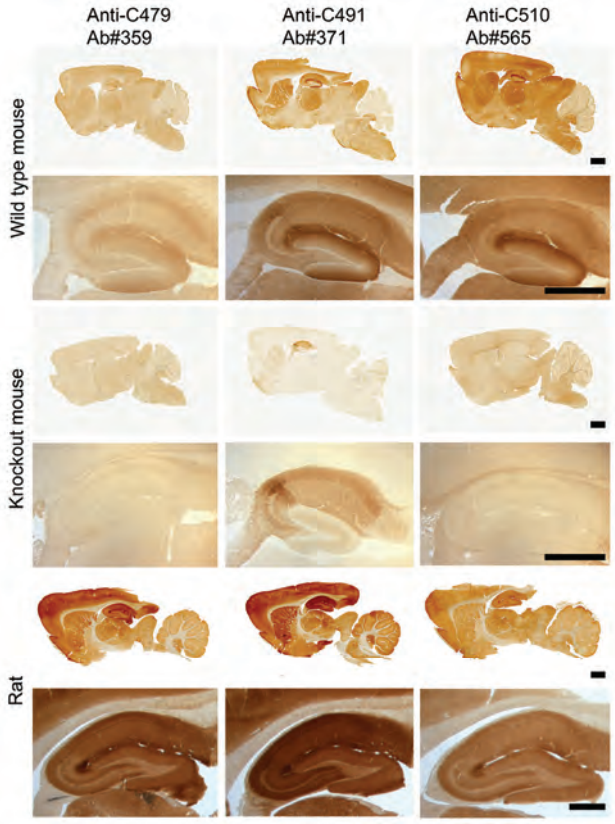


Figure 5. Changes in glutamate transporter expression during brain development. GLAST, GLT1, EAAC1 and EAAT4 protein expression were analyzed in rat forebrain and cerebellum tissue from E16 to adult age (two months). Note that the EAAC1 concentration in the forebrain is at its highest at P14. Three animals were tested at each age, but the figure only shows one of them. Abbreviations: E, embryonal day; P, postnatal day; A, adult. Each lane contained 20 µg protein and the blots were developed with antibody, anti-A522 (Ab#314; 0.2 µg/ml) for GLAST and anti-B12 (Ab#291; 0.2 µg/ml) for GLT1, anti-C491 (Ab#237; 1 µg/ml) for EAAC1 and anti-D537 (Ab#181; 1 µg/ml) for EAAT4.

Changes in EAAC1 concentrations during development

Rat brain tissue from E16 to adult was immunoblotted with antibodies to four different glutamate transporter subtypes. In contrast to the dramatic upregulation of GLAST and GLT1 in the hippocampus with age (Furuta et al., 1997; Ullensvang et al., 1997), only modest changes in the expression of EAAC1 were observed with age (Fig. 5). EAAC1 levels increased after birth and peaked at P14, before decreasing by a factor of three over the next six weeks. These results suggest that there is a transient increase in demand for EAAC1 activity during early postnatal life.



Antibody specificity on sections of aldehyde fixed tissue

Because intact fixed tissue might contain epitopes that are not present on immunoblots and vice versa, the antibodies were also tested on sections of wild-type and EAAC1-knockout mice (Fig. 6). As shown previously (Holmseth *et al.,* 2005, 2011), the anti-C491 antibodies recognize both rat and mice, while the anti-C479 antibodies produce only weak labeling of mouse sections and immunoblots (Fig. 1A3 vs. 1C3; Fig. 6). Most brain regions in EAAC1 deficient mice displayed very little labeling when compared to the same regions in the wild-type mice. However, the anti-

Figure 6. Diaminobenzidine-peroxidase labeling of brain sections from rat and mice (wild-type and EAAC1 deficient) with three different EAAC1 antibodies. High magnifications of hippocampus are shown below the overview pictures. Note that anti-C510 gave the best contrast between the wild-type and the knockout tissue. Anti-C479 produced only weak labeling in the mouse sections (like for immunoblots, as shown in figure 1C, lane 3), while anti-C491 produced some labeling in the knockout. Antibodies: anti-C479 (Ab#359; 10 µg/ml), anti-C491 (Ab#371; 0.3 µg/ml), and anti-C510 (Ab#565; 10 µg/ml). Triton X-100 (0.5 %) was included in the solutions. Rat and mouse tissue perfusion fixed with 4 % formaldehyde and 0.1% glutaraldehyde in 0.1 M sodium phosphate buffer. Rats were 14 weeks old, and mice 4 weeks old. Scale bar, 2 mm.

C491 antibodies (Ab#371) produced some labeling in EAAC1 deficient tissue (Fig. 6), although they appeared specific on immunoblots (Fig. 1 present study; Holmseth et al., 2011). Labeling in the knockout mice was observed in the hippocampus CA1-3 (CA2 in particular) and in the striatum as well as in some other places (e.g. piriform cortex). The identity of the cross-reacting molecule(s) was not determined, but it appears to have a cellular expression profile similar to EAAC1. The labeling in the EAAC1-deficient mice was only

seen in neurons (somato-dendritic compartment) and was thereby expressed in the same cells as those expressing EAAC1 (data not shown).

Note that the regional labeling patterns in mouse and rat obtained with the three antibodies are quite similar. As shown (Fig. 6) some differences in the hippocampus were noted, but these should not be over interpreted as it is difficult to get all parameters identical (animal age, fixation time, post fixation time, storage, time from sectioning to labeling, all of the assay conditions such as presence or absence of Triton X-100, etc.) and EAAC1-deficient rats were not available. Consequently, we cannot tell if these differences between mouse or rat are real. We did not pursue this question because the answer will have no impact on the conclusions of this study.

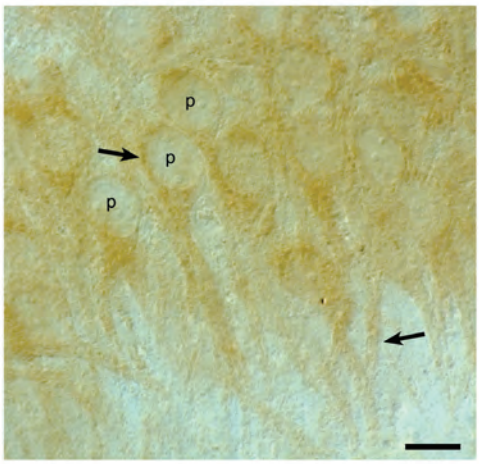


Figure 7. High magnification of hippocampus CA3 from EAAC1 wild-type mouse (4 weeks old) labeled with anti-C510 (Ab#565; 10 µg/ml). Note that cytoplasm in neuronal somata of pyramidal cells and dendrites are labeled (arrows). Pyramidal cell nuclei (p) are unlabeled (only three are indicated). The tissue was perfusion fixed with 4 % formaldehyde and 0.1% glutaraldehyde in 0.1 M sodium phosphate buffer and processed in the absence of Triton X-100. Scale bar=20 µm.

Cellular and subcellular localization of EAAC1

Vibratome sections of perfusion fixed rat brain were labeled with the various antibodies to EAAC1 in the absence of Triton X-100 using peroxidase as reporter. Light microscopy showed that labeling intensities varied between the various hippocampal subfields, with highest labeling observed in the dentate gyrus and the CA1 subfield. In these areas, we observed labeling of neuronal cell bodies and dendrites in both rats and wild-type mice with all the available anti-EAAC1 antibodies (Figs. 7, 8D). EAAC1 was found in all cells identified as neurons, including parvalbumin positive hippocampal interneurons (Fig. 8). In contrast, no labeling of endothelium was observed. Furthermore, the choroid plexus and the lining of the ventricles also were unlabeled, indicating that
EAAC1 was not expressed by ependymal cells or tanycytes.

We also did not detect EAAC1 in astrocytes in the adult or juvenile neocortex and hippocampus. Double labeling of sections with EAAC1 in combination with GLT1 (Fig. 9A), glial fibrillary acidic protein (GFAP, Fig. 9B), glutamine synthetase (GS, Fig. 9C, D, E, F, G)

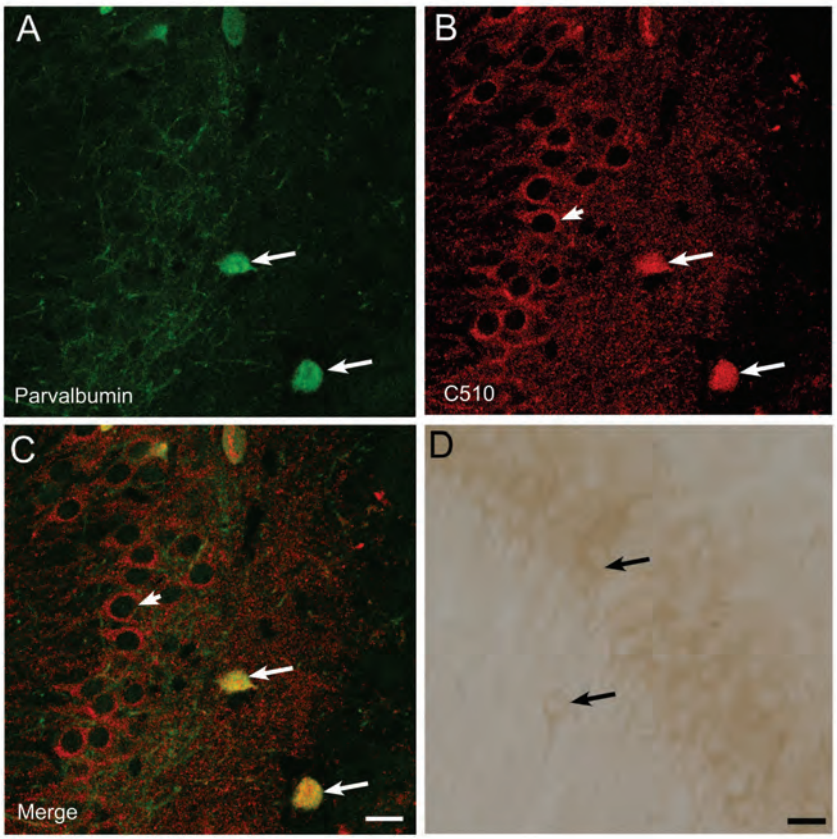


Figure 8. Hippocampal GABAergic interneurons expressing parvalbumin also express EAAC1. Panels A-C: (A, green) anti-parvalbumin (1:500), (B, red) anti-EAAC1 (anti-C510; Ab#565, 10 µg /ml) and (C) a merged picture of the two. Panel D: anti-EAAC1 (anti-C510; Ab#565, 10 µg /ml) developed with the diaminobenzidine-peroxidase labeling system. Note that both EAAC1 and parvalbumin are expressed in cell bodies in stratum pyramidale (arrow head) and stratum radiatum (arrows). The tissue in A-C is from mouse, 3 weeks old perfusion fixed with 4 % formaldehyde in 0.1 M NaPi. D is form a 3 weeks old mouse perfusion fixed with 4 % formaldehyde and 0.05 % glutaraldehyde in 0.1 M NaPi. Scale bars, 20 µm

or GLAST (Fig. 9H) did not reveal any co-localization between EAAC1 and astrocytic markers. Two different GLT1 antibodies were used as a positive control for co-localization (Fig. 9I). In the corpus callosum there also was no co-localization of EAAC1 with markers for mature oligodendrocytes (myelin basic protein, MBP; CNPase), and the EAAC1-immunoreactive cells were scattered rather than organized in string-like arrays typical of oligodendrocytes (Fig. 10). EAAC1 immunoreactivity also did not co-localize with NG2, indicating that this transporter is not expressed by oligodendrocyte precursor cells (NG2 cells) or pericytes. This is in agreement with a previous report (Berger and Hediger, 1998) where it was suggested that EAAC1 immunoreactive cells in the corpus callosum represent

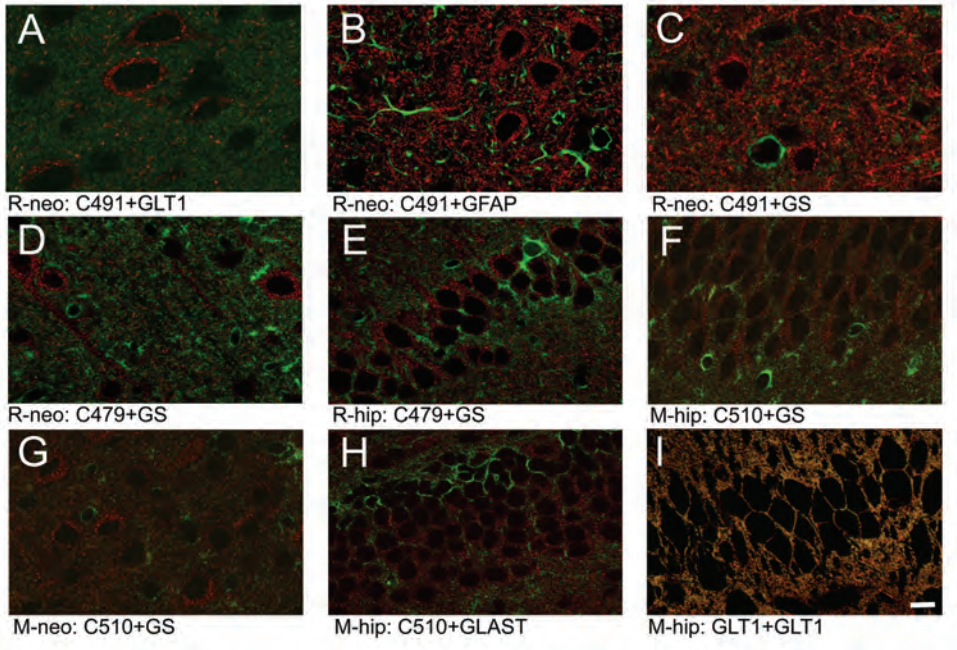
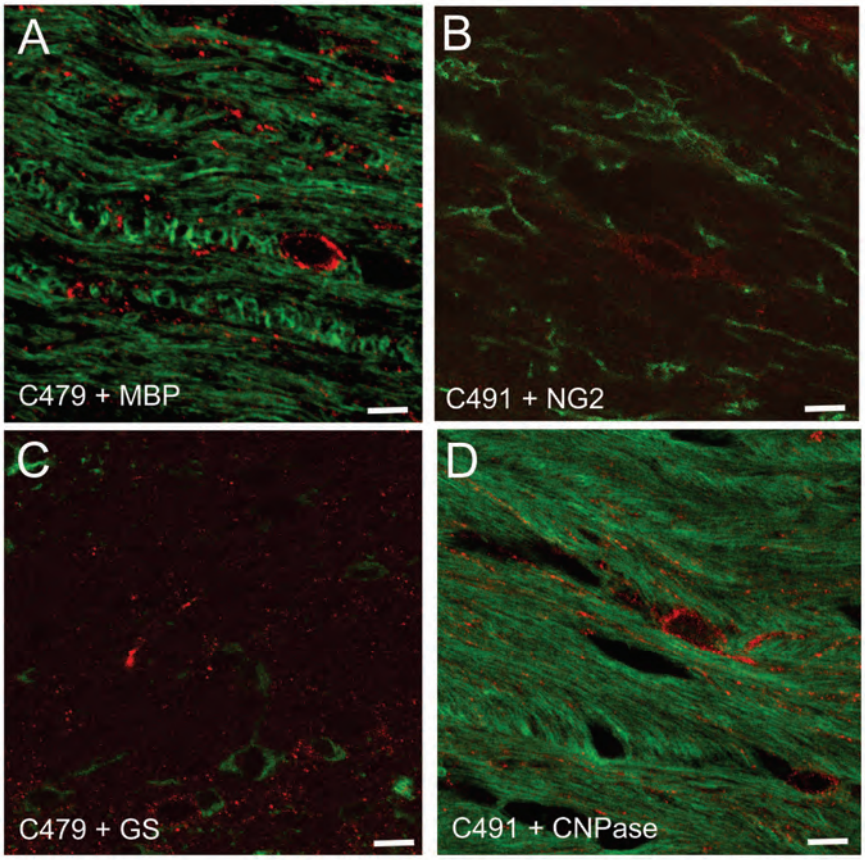


Figure 9. Double labeling with three different EAAC1 antibodies (red) and different astrocyte markers (green). In A-C anti-C491 (Ab#371, 0.5 µg /ml) is used together with the following markers: GLT1 (A, 1:500), glial fibrillary acidic protein (GFAP) (B, 1:500), and glutamine synthetase (GS) (C, 1:500) in rat neocortex. In D-E anti-C479 (Ab#359, 3 µg /ml) is used together with glutamine synthetase in rat neocortex (D) and in rat hippocampus CA1 (E). In F-H anti-C510 (Ab#565, 30 µg /ml) is used together with glutamine synthetase (F-G) or anti-GLAST (Banner et al., 2002) 1:500 (H) as indicated. F and H are from mouse hippocampus CA1, while G is from mouse neocortex. Double labeling with mouse anti-GLT1 (9C4; Ab#531, 1:30) and rabbit anti-GLT1 (Ab#360, 0.5 µg /ml) is a positive control for co-localization (I). Note that there is no significant co-localization between EAAC1 and the various astroglial markers. Rat and mouse tissue perfusion fixed with 4 % formaldehyde in 0.1 M Sodium phosphate buffer. Rats were 9 weeks old, and mice 3 weeks old. Abbreviations: M-hip, mouse hippocampus; M-neo, mouse neocortex; R-hip, rat hippocampus; R-neo, rat neocortex. Scale bars, 10 µm.

interneurons. In contrast to the strong immunoreactivity observed in the soma and dendrites of neurons, there was no detectable co-localization between EAAC1 and markers for nerve terminals in the neocortex, hippocampus, or cerebellar nuclei. As shown (Fig. 11), EAAC1 antibodies did not label the structures that were labeled with antibodies to synaptophysin, GAD and VGLUT1. It is clear from these sections that EAAC1 labeling was largely intracellular in the brain. This is in contrast to the kidney where EAAC1 is concentrated at the cell surface, particularly in the brush border of proximal tubules (Fig. 12).

The EAAC immunoreactivity observed electron microscopically (Fig. 13) was weak in agreement with figure 7. The very strong labeling that we have previously observed with



a

Figure 10. Double labeling with various EAAC1 antibodies (red) and markers for oligodendrocytes (green) in corpus callosum from adult mouse. In A and C anti-C479 (Ab#359, 3 µg/ml) is used together with the anti-myelin basic protein (MBP) (A, 1:500) and anti-glutamine synthetase (GS, C, 1:500). In B and D anti-C491 (Ab#371, 0.5 µg /ml) is used together with anti-NG2 (B, 1:500) and anti-CNPase (D, 1:500). Note that there is no detectable co-localization with any of the glial markers. Scale bar: 10 µm.

antibodies to GLT1 and GLAST (e.g. Danbolt et al., 1992; Levy et al., 1993; Chaudhry et al., 1995; Lehre et al., 1995) and to EAAT4 in the cerebellum (Dehnes et al., 1998) was not only due to good antibodies and good tissue processing, but also due to the very high expression levels of these proteins (Lehre and Danbolt, 1998). With GLT1 antibodies and gold-labeled secondary antibodies, we observed on average, when using the best antibodies, around 10 gold particles per micrometer membrane length, and background labeling was not a problem (Chaudhry et al., 1995; Furness et al., 2008). Considering that EAAC1 is expressed at levels two order of magnitude lower, it is not surprising that the immunogold labeling was similar to the background level observed in the EAAC1-knockout tissue. In fact, if the labeling is specific for EAAC1, then this is exactly what should be expected.

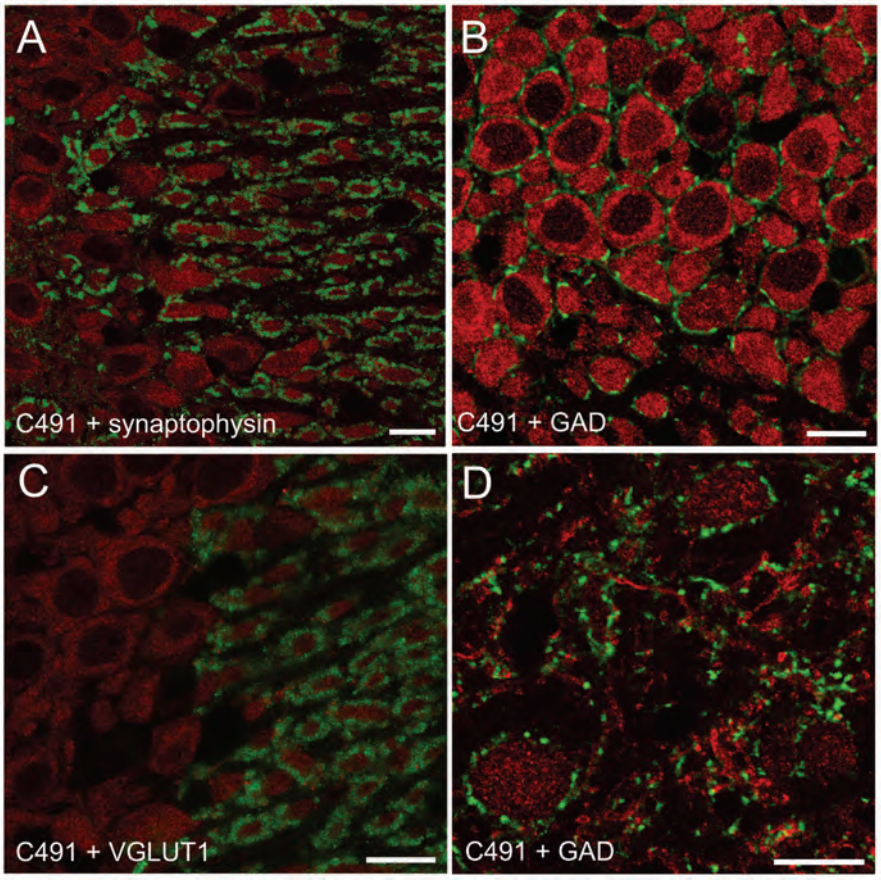


Figure 11. Double labeling with EAAC1 (red) and various markers for nerve terminals (green). A-D shows EAAC1 labeled with anti-C491 (Ab#371, 1 µg /ml) together with anti-synaptophysin (1:1000) (A), anti-GAD (1:500) (B and D), and anti-VGLUT1 (1:200). Note that there is no detectable co-localization with any of the markers for terminals. Pictures A-C are from hippocampus CA1, while D is from the cerebellar nuclei. Rat tissue (9 weeks) was perfusion fixed with 4 % formaldehyde in 0.1 M sodium phosphate buffer. Scale bars, 20 µm.axons, or glial cell processes. Although there was some labeling of the outer membranes of mitochondria, this is likely to be an artifact, because mitochondria have been shown not to contain EAAC1 (Holmseth et al., 2006). Together, these studies show that EAAC1 is predominantly intracellular, but is expressed at such low levels that it is close to the detection limit using immunolabeling approaches suitable for electron microscopy. Immunofluorescence and confocal microscopy is more sensitive (but has lower resolution).

The EAAC1 labeling that we did detect, was intracellularly in perikaria and dendrites, where it was associated with structures resembling vesicular clusters and patches of plasma membranes (Fig. 13). No labeling was observed in structures clearly identified as terminals,

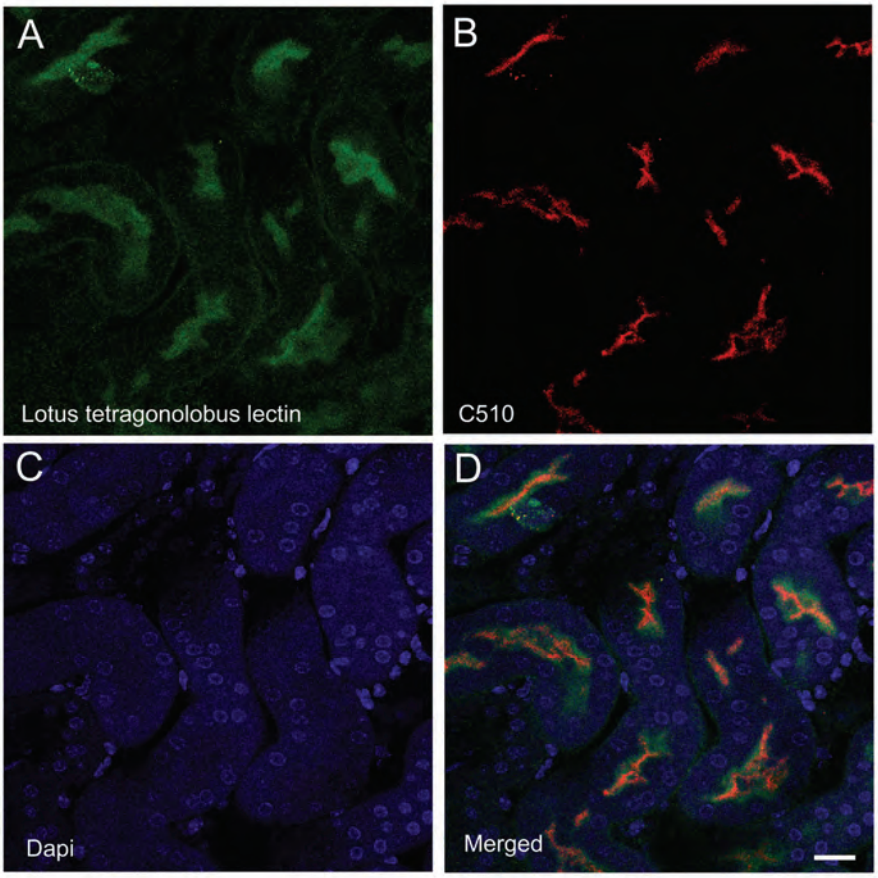


Figure 12. Double labeling with EAAC1 (red) and a marker for renal proximal tubules (*Lotus tetragonolobus* lectin; green). A shows *Lotus tetragonolobus* lectin (1:300), B shows EAAC1 labeled with anti-C510 (Ab#565, 3 µg/ml), C shows cell nuclei (DAPI staining) and D is a merged picture of the three. Note that EAAC1 is concentrated in the brush border of proximal tubules. Mouse tissue (12 weeks) was perfusion fixed with 4 % formaldehyde and 0.1 % glutaraldehyde in 0.1 M sodium phosphate buffer. Scale bar, 10 µm.

axons, or glial cell processes. Although there was some labeling of the outer membranes of mitochondria, this is likely to be an artifact, because mitochondria have been shown not to contain EAAC1 (Holmseth *et al.*, 2006). Together, these studies show that EAAC1 is predominantly intracellular, but is expressed at such low levels that it is close to the detection limit using immunolabeling approaches suitable for electron microscopy. Immunofluorescence and confocal microscopy is more sensitive (but has lower resolution).

The number of EAAC1 molecules per square micrometer plasma membrane

As shown previously (Furness *et al.*, 2008), the most important factor contributing to surface densities is whether or not a protein is expressed in axons and terminals because these

structures account for about three quarters of the total plasma membranes in stratum radiatum of hippocampus CA1. The dendritic surface area in this area is 1.5 $\mu m^2/\mu m^3$, similar to the surface area of spines in the molecular layer of the cerebellum (1.1 $\mu m^2/\mu m^3$) (Dehnes et al., 1998). The astroglial surface area, which was included as a control, is 1.4 $\mu m^2/\mu m^3$, and thus identical to our previous analysis using a different stereological method (Lehre and Danbolt, 1998). Given the measured EAAC1concentrations (Tables 2 and 3), the mean EAAC1 density is about 90 molecules per square micrometer of dendritic membrane, provided all EAAC1 is inserted into the membrane. However, as most of the EAAC1 is located intracellularly, this is a significant overestimation. The above calculations therefore assume that EAAC1 is evenly distributed. As can be seen in Fig. 6 this is an approximation.

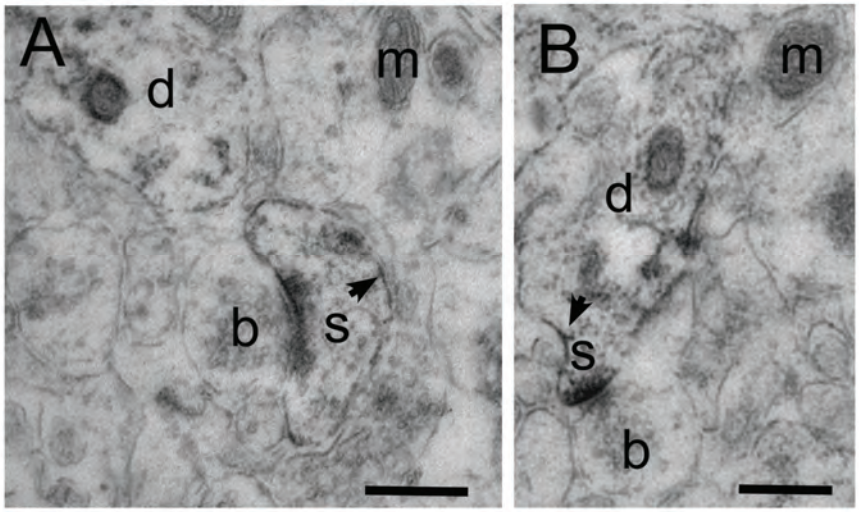


Figure 13. Electron micrographs from dentate gyrus molecular layer of wild-type mouse showing postsynaptic neuronal EAAC1 labeling. Labeling is found in dendritic compartments (d), including spines (s). Arrowheads point at labeling at the cell membrane in spines. Note that the labeling appears more patchy than the astrocytic GLT and GLAST membrane labeling produced with the same method in our previous studies (Lehre et al., 1995). Mitochondria (m) are indicated. Boutons (b) appear unlabelled. Antibody: anti-C510 (Ab#565; 10 µg/ml). Mouse tissue (4 weeks) was perfusion fixed with 4 % formaldehyde and 0.05 % glutaraldehyde in 0.1 M sodium phosphate buffer. Scale bars, 300 nm.

Table 3. Tissue concentrations of glutamate transporters in the hippocampus (stratum radiatum, CA1) of young adult Wistar rats

	mg per g tissue	μM	Molecules per μm^3	$\mu m^2/\mu m^3$	Location	Percent the surface	Molecules per μm^2
GLAST[1]	0.32	5.3	3200	1.4	Astroglia	~100 %	2300
GLT1[1]	1.3	21	12000	1.4	Astroglia[2]	~100 %	7500[2]
					Terminals	~10 %	750
EAAC1	0.013	0.22	130	1.5	Dendrites	0-100 %	0-90[3]

The above data only give an indication of the average transporter densities because expression levels are subject to regulation. Further, EAAC1 is to a large extent present intracellularly in the brain (e.g. Figs. 7 and 8) while most of GLT1 and GLAST are at the surface. The calculations further assume that brain tissue contains about 97.8 g protein per Kg (Lowry et al., 1954) and has a density of 1.05 g/cm^3 (Lowry, 1953) (i.e. 1 μm^3 contains about 10^{-13}g protein), and that the molecular masses of the polypeptide parts of rat EAAT1 (Storck et al., 1992), EAAT2 (Pines et al., 1992) and EAAC1 (Kanai and Hediger, 1992) are 60, 62 and 58 kDa, respectively. The total plasma membrane density was about 14 $\mu m^2/\mu m^3$. Axons represents about 70 % of this while astroglia, butons, dendrites including spines represent about 10 % each (Furness *et al.*, 2008).

[1] Data from Lehre and Danbolt, 1998.

[2] It is now clear that about 10% of the EAAT2 is also present in synaptic terminals (Furness *et al*, 2008) implying that the number 8500 given in Lehre and Danbolt, 1998 is slightly too high.

[3] A range is given because it is unclear how much is in the membrane.

Lack of detectable glutamate transporter currents in hippocampal CA1 pyramidal neurons

To determine whether the surface density of EAAC1 in hippocampal neurons is sufficient to produce detectible transporter currents, we made whole cell voltage clamp recordings from CA1 pyramidal neurons using solutions appropriate to maximize transporter current amplitude. Although photolysis of caged D-aspartate (MNI-D-aspartate), a substrate of glutamate transporters, reliably elicited large inward currents in hippocampal astrocytes (–415 ± 67 pA, n = 6) (Fig. 14A) and cerebellar Purkinje neurons (–634 ± 63 pA, n = 8) (Fig 14B) in rat CNS tissue, which were inhibited by the glutamate transporter antagonist DL-threo-β-benzyloxyaspartate (TBOA; 200 μM), only small responses were observed in hippocampal pyramidal neurons (–9 ± 2 pA, n = 7) (Fig 14C1). These small currents were not sensitive to TBOA, and responses with similar kinetics were observed in both wild-type (–6 ± 1 pA, n = 3) and EAAC1-deficient mice (–5 ± 1 pA, n = 5; P > 1), indicating that they did not arise from cycling of EAAC1. These findings are in agreement with earlier studies which found that evoked release of glutamate from Schaffer collateral fibers in rat did not elicit transporter currents in CA1 pyramidal neurons (Bergles and Jahr, 1998), providing further support for the conclusion that few functional EAAC1 transporters are present at the cell surface of these neurons.

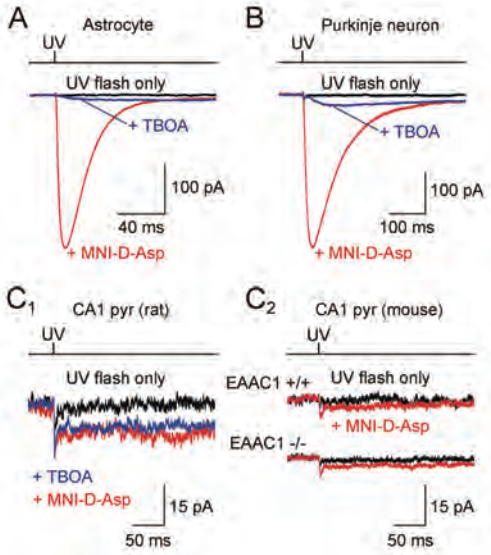
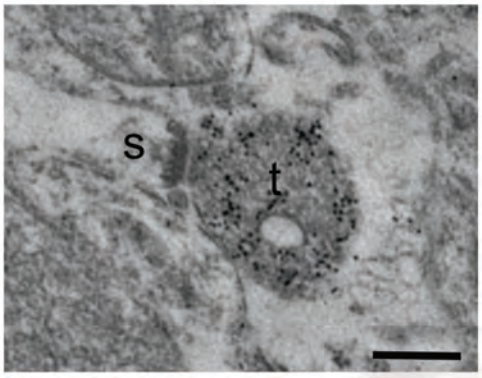


Figure 14. Glutamate transporter currents are visible in hippocampal astrocytes and cerebellar Purkinje neurons, but not hippocampal CA1 pyramidal neurons. (A) A transient inward current was elicited in an astrocyte by a 1 ms flash of UV laser light. The inward current was observed only in the presence of MNI-D-aspartate (MNI-D-Asp, 125 µM), and was inhibited by TBOA (200 µM). The trace above shows the duration of UV exposure. Recordings were made from astrocytes located in stratum radiatum of area CA1, in the presence of TTX (1 µM), R,S-CPP (10 µM), MK-801 (50 µM), NBQX (10 µM) and SR95531 (5 µM). Astrocytes were voltage-clamped at –80 mV with a KMeSbased internal solution. (B) Glutamate transporter currents evoked in Purkinje neurons through photolysis of MNI-D-aspartate (500 µM), in the absence and presence of TBOA (200 µM). Cesium nitrate ($CsNO_3$)-based internal solution. (C) Glutamate transporter currents were not observed in hippocampal CA1 pyramidal neurons. (C1) A small inward current was evoked in a rat hippocampal CA1 pyramidal neuron in response to photolysis of MNI-D-aspartate (500 µM). The inward current was slightly larger in the presence than in the absence of MNI-D-aspartate; however, this response was not inhibited by TBOA (200 µM). (C2) Photolysis-evoked currents from CA1 pyramidal neurons in slices from wild-type (EAAC1+/+) and EAAC1 deficient (EAAC1-/-) mice, showing no significantly larger current in wild-type mice. Recordings were made in the presence of TTX (1 µM), R,S-CPP (10 µM), MK-801 (50 µM), 5,7-Dichlorolkynurenic acid (20-100 µM), NBQX (10 µM) and SR95531 (5 µM). Pyramidal neurons were voltage-clamped at -65 mV with a potassium thiocyanate-based internal solution.



D-Aspartate uptake in nerve terminals in rat hippocampal slices

To increase the sensitivity of detection, we attempted to visualize EAAC1 activity in spines using immunocytochemistry. D-aspartate was chosen as a transporter substrate because this molecule is poorly metabolized in brain tissue (Davies and Johnston, 1976; Takagaki, 1978). Hippocampal slices were incubated with D-aspartate, fixed, embedded, cut, immunolabeled and studied electron microscopically in the stratum radiatum

Figure 15. Glutamate uptake in spines is relatively low, as demonstrated by D-Aspartate uptake and immunocreactivity. Hippocampal slices form adult rat (9 weeks old) were incubated in D-aspartate (50 µM, 3 min), fixed (1 % formaldehyde and 2.5 % glutaraldehyde) and immunogold labeled with antibodies to glutaraldehyde-fixed D-aspartate. Labeling shows accumulation of D aspartate in.nerve terminals (t) - (there is a

high number of black uniformly sized dots representing gold-particles over the terminal). Glia (not shown) were also heavily labeled. Dendrites and dendritic spines (s) were virtually unlabeled. Note that there are no gold-particles over the spine (s). Arrowheads indicate the location of a synaptic cleft. Scale bar, 300 nm.

of area CA1. D-aspartate immunoreactivity was observed in synaptic terminals (Fig. 15) and astroglia (not shown). Despite heavy labeling of these structures, no labeling of spines was observed (Fig. 15). Hippocampal slices incubated without D-aspartate had very little immunoreactivity, while slices incubated with D-aspartate in combination with an uptake inhibitor (L-glutamate or dihydrokainate) were weakly positive (data not shown; see Furness et al., 2008). Thus, the uptake activity in spines is negligible compared to that present in other synaptic compartments.

DISCUSSION

EAAC1 has been intensely studied, but experimental data has been interpreted quite differently; in part because quantitative data on EAAC1 protein levels have been lacking.

EAAC1 is exclusively expressed by neurons throughout the CNS

In agreement with other reports on EAAC1 distribution (for review see: Danbolt, 2001), we show that EAAC1 is present in the cell bodies and dendrites of most, if not all, neurons in the forebrain. Also in agreement with many (Kanai and Hediger, 1992; Rothstein et al., 1994; Torp et al., 1997; Berger and Hediger, 1998; Coco et al., 1997; Shashidharan et al., 1997; Plachez et al., 2000; Holmseth et al., 2005), but not all (e.g. Conti et al., 1998; Kugler and Schmitt, 1999), we do not find evidence for expression in astrocytes and oligodendrocytes.

Is EAAC1 in terminals?

Another unresolved issue is the reported pre-synaptic expression of EAAC1. Rothstein and co-workers (Rothstein et al., 1994) did not find EAAC1 in glutamatergic terminals, but noted that GABAergic terminals in the deep cerebellar nuclei were labeled. Other studies (He et al., 2000; He et al., 2001) reported that EAAC1 is present in both GABAergic and glutamatergic terminals, and found about 10 % of the labeling in the latter. However, these investigators did not have access to tissue from the EAAC1-deficient mice to verify the labeling specificity, and their conclusions are at variance with other reports (e.g. Shashidharan et al., 1997; Conti et al., 1998; Holmseth et al., 2005) as well as with our data (Fig. 11). Further, in agreement with Rothstein (1994) we find no glutamate uptake activity in glutamatergic terminals in GLT1-deficient mice (Furness *et al.*, 2008). In conclusion, we are unable to support the notion that there are functionally relevant levels of EAAC1 in glutamatergic terminals.

In GABAergic terminals, however, the situation is more complex. A study based on antisense knockdown of EAAC1 concluded that EAAC1 is important for GABA synthesis in GABAergic terminals (Sepkuty et al., 2002). It can be argued that antisense probes may have unrecognized effects, but there is other functional evidence for glutamate uptake into GABAergic terminals (Mathews and Diamond, 2003; Stafford et al., 2010). The latter studies show that raising the extracellular glutamate levels, increases mIPSC amplitudes possibly because EAAC1-mediated glutamate uptake in GABAergic terminals provides glutamate for GABA synthesis increasing GABA levels. However, Stafford and co-workers (2010), in agreement with our data, failed to detect EAAC1 protein in terminals directly. In stead, they incubated slices in D-aspartate (250 μM; 30 min) and double-labeled these with antibodies to

D-aspartate and GAD. By this method they showed that D-aspartate can be taken up by GABAergic terminals. This is in principle a sensitive technique because each transporter can transport a huge number of D-aspartate molecules in 30 minutes. However, the D-aspartate concentration used was 30 times higher than Km (Km for D-aspartate is 7.5 μM; Kanai and Hediger, 1992). This weakens the argument because maximum EAAC1 mediated uptake should be observed at lower concentrations. Thus, it can be argued that the observed uptake is due to other transporters. Nevertheless, the authors conclude that EAAC1 is likely there, at densities below the threshold for direct detection. The limit for direct detection with antibodies is unknown, but a fair guess with our antibodies is that the limit is at around 10 % of the average density observed in hippocampal spines. If so, the possibility exists that up to dozen EAAC1 molecules in each GABAergic terminal may have evaded our detection, but this is probably not enough EAAC1 molecules to account for the observed increased mIPSC amplitudes.

Cell surface expression of EAAC1

In order for transporters to participate in glutamate clearance, they must be localized to the plasma membrane. GLT1, GLAST (Lehre and Danbolt, 1998) and EAAT4 (Dehnes et al., 1998) are highly enriched at the cell surface, and transporter-mediated currents can be recorded from astrocytes (Bergles and Jahr, 1997; Huang et al., 2004) and Purkinje cells (Otis et al., 1997) in response to application of substrates, supporting a role for these transporters in glutamate clearance. In contrast, EAAC1 immunoreactivity was predominantly intracellular, where it was often associated with vesicle-like structures. It could be that proteins anchoring EAAC1 at the surface mask the epitope making EAAC1 at the cell surface invisible. This is, however, unlikely because similar results were obtained by an antibody to another EAAC1 region (Shashidharan et al., 1997) and because EAAC1 was readily detected in the brush border in the kidney. Further, cell culture studies show that only 20-30% of EAAC1 is localized at the plasma membrane (Fournier et al., 2004; Sheldon et al., 2006), and we were unable to record EAAC1-mediated transporter currents from neurons (see also Bergles and Jahr, 1998).

The contribution of EAAC1 to synaptic glutamate clearance

As shown here (Table 3), the mean EAAC1 density (in hippocampus of young adult rats) was approximately 90 transporters per square micrometer of dendritic membrane, provided all EAAC1 is inserted into the membrane, and about 130 molecules per synapse. As several thousand molecules of glutamate are released from each vesicle, it appears unlikely that there are enough EAAC1 transporters to have an appreciable effect on glutamate clearance in the brain, in agreement with our electrophysiological results, i.e. the lack of detectable glutamate uptake currents in hippocampal pyramidal cells and the lack of significant differences between such currents in pyramidal neurons from wild-type and EAAC1-knockout mice. Further, the low glutamate uptake activity in the GLT1-deficient mice (Fig. 4) and the low uptake activity in spines (Fig. 15; see also Furness et al., 2008) also point to a negligible contribution from EAAC1. Our findings are, however, at variance with reports based on antisense knockdown of transporters (Rothstein et al., 1996). A possible explanation for this is unrecognized side effects of the antisense probes causing an overestimation of the importance of EAAC1.

The results presented here suggest that GLT1 represents about 80 % of all glutamate transporter proteins in the young adult hippocampus, with GLAST comprising most of the

remaining 20 %, and EAAC1 representing only about 1 %. Because GLAST and EAAC1 are about 6 and 1.5 times slower than GLT1 (Grewer and Rauen, 2005), respectively, it follows that the effective contribution to uptake activity is dominated by GLT1 (> 95 %) in agreement with previous data (Haugeto et al., 1996; Tanaka et al., 1997).

Physiological roles of EAAC1 in the brain

EAAC1 appears to be the only glutamate transporter expressed by most mature neurons. As it also transports cysteine, it seems likely that EAAC1 does serve metabolic roles as suggested (Aoyama et al., 2006). As explained above, EAAC1 is not likely to contribute to the maintenance of low resting levels of glutamate.

However, in the absence of an EAAC1 selective antagonist, it is difficult to assess more sophisticated functions of this transporter in the brain. Scimemi and co-workers (2009) addressed this question and described functional differences in hippocampal slices from wild-type and EAAC1-knockout mice. They suggest that EAAC1 buffers glutamate released during synaptic events and prolongs the time course of its clearance by astrocytes. Without significantly altering activation of receptors in the synaptic cleft, EAAC1 reduces recruitment of perisynaptic/extrasynaptic NR2B-containing NMDARs, thereby facilitating induction of long-term potentiation by short bursts of high-frequency stimulation. This is an unusually extensive and impressive study. As far as we can see, they have taken into account all factors known to be relevant. However, their own 3D Monte Carlo modeling only matches their experimental data if EAAC1 is less efficient than GLT1, and if EAAC1 is present in numbers that are much higher than those we report here. It therefore seems likely that the functional differences between wild-type and EAAC1-knockout mice (Scimemi et al., 2009) are caused by as yet unidentified downstream consequences of the loss of EAAC1, possibly in combination with other factors such as the trauma induced by the slicing of brain tissue or by the subsequent *in vitro* treatment. After all, tissue ultrastructure, such as astrocytic coverage of synapses, is modulated by neuronal activity (e.g. Genoud et al., 2006), and the ultrastructure of hippocampal slice preparations is affected by the method of preparation and is quite different from that of hippocampus fixed *in vivo* by cardiac perfusion (e.g. Fiala et al., 2003). Although Scimemi and co-workers (2009) have considered these factors and also have considered impaired glutathione synthesis (Aoyama et al., 2006), there may yet be unidentified differences between wild-type and EAAC1-deficient mice.

Conclusions

EAAC1 is present in cell bodies and dendrites of most, if not all, neurons. It was not detected in terminals, neither glutamatergic nor GABAergic. Further, EAAC1 was not detected in astrocytes, oligodendrocytes or endothelial cells. The concentration of EAAC1 in the young adult hippocampus is two orders of magnitude lower than that of GLT1. The localization and the low expression levels argue against a role in glutamate clearance and buffering. EAAC1 is more likely to play metabolic roles considering that it can transport both glutamate and cysteine, and that it is the only glutamate transporter expressed by most neurons.

REFERENCES

Aoyama K, Suh SW, Hamby AM, Liu J, Chan WY, Chen Y, Swanson RA (2006) Neuronal glutathione deficiency and age-dependent neurodegeneration in the EAAC1 deficient mouse.

Nat Neurosci 9:119- 126.
Bailey CG, Ryan RM, Thoeng AD, Ng C, King K, Vanslambrouck JM, Auray-Blais C, Vandenberg RJ, Bröer S, Rasko JE, Weinstein N, Hodgins HS, Ryan RM (2011) Loss-of-function mutations in the glutamate transporter SLC1A1 cause human dicarboxylic aminoaciduria. J Clin Invest 121:446-453.
Banner SJ, Fray AE, Ince PG, Steward M, Cookson MR, Shaw PJ (2002) The expression of the glutamate re-uptake transporter excitatory amino acid transporter 1 (EAAT1) in the normal human CNS and in motor neuron disease: an immunohistochemical study. Neuroscience 109:27-44.
Beckstrøm H, Julsrud L, Haugeto Ø, Dewar D, Graham DI, Lehre KP, Storm-Mathisen J, Danbolt NC (1999) Interindividual differences in the levels of the glutamate transporters GLAST and GLT, but no clear correlation with Alzheimer's disease. J Neurosci Res 55:218-229.
Berger UV, Hediger MA (1998) Comparative analysis of glutamate transporter expression in rat brain using differential double in situ hybridization. Anat Embryol (Berl) 198:13-30.
Bergles DE, Jahr CE (1997) Synaptic activation of glutamate transporters in hippocampal astrocytes. Neuron 19:1297-1308.
Bergles DE, Jahr CE (1998) Glial contribution to glutamate uptake at schaffer collateral-commissural synapses in the hippocampus. J Neurosci 18:7709-7716.
Berman AE, Chan WY, Brennan AM, Reyes RC, Adler BL, Suh SW, Kauppinen TM, Edling Y, Swanson RA (2011) N-acetylcysteine prevents loss of dopaminergic neurons in the EAAC1(-/-) mouse. Ann Neurol 69:509-520.
Bjørås M, Gjesdal O, Erickson JD, Torp R, Levy LM, Ottersen OP, Degree M, Storm-Mathisen J, Seeberg E, Danbolt NC (1996) Cloning and expression of a neuronal rat brain glutamate transporter. Brain Res Mol Brain Res 36:163- 168.
Chaudhry FA, Lehre KP, Campagne MV, Ottersen OP, Danbolt NC, Storm-Mathisen J (1995) Glutamate transporters in glial plasma membranes: highly differentiated localizations revealed by quantitative ultrastructural immunocytochemistry. Neuron 15:711-720.
Chen YM, Swanson RA (2003) The glutamate transporters EAAT2 and EAAT3 mediate cysteine uptake in cortical neuron cultures. J Neurochem 84:1332- 1339.
Coco S, Verderio C, Trotti D, Rothstein JD, Volterra A, Matteoli M (1997) Non-synaptic localization of the glutamate transporter EAAC1 in cultured hippocampal neurons. Eur J Neurosci 9:1902-1910.
Conti F, DeBiasi S, Minelli A, Rothstein JD, Melone M (1998) EAAC1, a high-affinity glutamate transporter, is localized to astrocytes and GABAergic neurons besides pyramidal cells in the rat cerebral cortex. Cereb Cortex 8:108- 116.
Danbolt NC (2001) Glutamate uptake. Prog Neurobiol 65:1-105.
Danbolt NC, Lehre KP, Dehnes Y, Chaudhry FA, Levy LM (1998) Localization of transporters using transporter-specific antibodies. Methods Enzymol 296:388-407.
Danbolt NC, Pines G, Kanner BI (1990) Purification and reconstitution of the sodium- and potassium-coupled glutamate transport glycoprotein from rat brain. Biochemistry 29:6734-6740.
Danbolt NC, Storm-Mathisen J, Kanner BI (1992) An [Na^++ K^+]coupled L-glutamate transporter purified from rat brain is located in glial cell processes. Neuroscience 51:295-310.
Davies LP, Johnston GA (1976) Uptake and release of D- and L-aspartate by rat brain slices. J Neurochem 26:1007-1014.
Dehnes Y, Chaudhry FA, Ullensvang K, Lehre KP, Storm-Mathisen J, Danbolt NC (1998) The

glutamate transporter EAAT4 in rat cerebellar Purkinje cells: a glutamate- gated chloride channel concentrated near the synapse in parts of the dendritic membrane facing astroglia. J Neurosci 18:3606-3619.

Fiala JC, Kirov SA, Feinberg MD, Petrak LJ, George P, Goddard CA, Harris KM (2003) Timing of neuronal and glial ultrastructure disruption during brain slice preparation and recovery in vitro. J Comp Neurol 465:90-103.

Fournier KM, Gonzalez MI, Robinson MB (2004) Rapid trafficking of the neuronal glutamate transporter, EAAC1 - Evidence for distinct trafficking pathways differentially regulated by protein kinase C and platelet-derived growth factor. J Biol Chem 279:34505- 34513.

Furness DN, Dehnes Y, Akhtar AQ, Rossi DJ, Hamann M, Grutle NJ, Gundersen V, Holmseth S, Lehre KP, Ullensvang K, Wojewodzic M, Zhou Y, Attwell D, Danbolt NC (2008) A quantitative assessment of glutamate uptake into hippocampal synaptic terminals and astrocytes: new insights into a neuronal role for excitatory amino acid transporter 2 (EAAT2). Neuroscience 157:80-94.

Furuta A, Rothstein JD, Martin LJ (1997) Glutamate transporter protein subtypes are expressed differentially during rat CNS development. J Neurosci 17:8363-8375.

Genoud C, Quairiaux C, Steiner P, Hirling H, Welker E, Knott GW (2006) Plasticity of astrocytic coverage and glutamate transporter expression in adult mouse cortex - art. no. e343. PLoS Biol 4:2057-2064.

Grewer C, Rauen T (2005) Electrogenic glutamate transporters in the CNS: molecular mechanism, pre-steady-state kinetics, and their impact on synaptic signaling. J Membr Biol 203:1-20.

Haugeto Ø, Ullensvang K, Levy LM, Chaudhry FA, Honoré T, Nielsen M, Lehre KP, Danbolt NC (1996) Brain glutamate transporter proteins form homomultimers. J Biol Chem 271:27715- 27722.

He Y, Hof PR, Janssen WG, Rothstein JD, Morrison JH (2001) Differential synaptic localization of GluR2 and EAAC1 in the macaque monkey entorhinal cortex: a postembedding immunogold study. Neurosci Lett 311:161-164.

He Y, Janssen WG, Rothstein JD, Morrison JH (2000) Differential synaptic localization of the glutamate transporter EAAC1 and glutamate receptor subunit GluR2 in the rat hippocampus. J Comp Neurol 418:255- 269.

Holmseth S, Dehnes Y, Bjørnsen LP, Boulland JL, Furness DN, Bergles D, Danbolt NC (2005) Specificity of antibodies: unexpected cross reactivity of antibodies directed against the EAAT3 (EAAC) glutamate transporter. Neuroscience 136:649-660.

Holmseth S, Lehre KP, Danbolt NC (2006) Specificity controls for immunocytochemistry. Anat Embryol (Berl) 211:257- 266.

Holmseth S, Scott HA, Real K, Lehre KP, Leergaard TB, Bjaalie JG, Danbolt NC (2009) The concentrations and distributions of three C-terminal variants of the GLT1 (EAAT2; slc1a2) glutamate transporter protein in rat brain tissue suggest differential regulation. Neuroscience 162:1055-1071.

Holmseth S, Zhou Y, Follin-Arbelet VV, Lehre KP, Bergles DE, Danbolt NC (2011) Specificity controls for immunocytochemistry: the antigen pre-absorption test can lead to inaccurate assessment of antibody specificity. Submitted.

Huang YH, Sinha SR, Fedoryak OD, Ellis-Davies GC, Bergles DE (2005) Synthesis and Characterization of 4- Methoxy-7-Nitroindolinyl-D-Aspartate, a Caged Compound for Selective Activation of Glutamate Transporters and N-Methyl-D-Aspartate Receptors in Brain Tissue. Biochemistry 44:3316-3326.

Huang YH, Sinha SR, Tanaka K, Rothstein JD, Bergles DE (2004) Astrocyte glutamate transporters regulate metabotropic glutamate receptor-mediated excitation of hippocampal Interneurons. J Neurosci 24:4551-4559.

Kanai Y, Hediger MA (1992) Primary structure and functional characterization of a high-affinity glutamate transporter. Nature 360:467- 471.

Kugler P, Schmitt A (1999) Glutamate transporter EAAC1 is expressed in neurons and glial cells in the rat nervous system. Glia 27:129-142.

Lehre AC, Rowley NM, Zhou Y, Holmseth S, Guo C, Holen T, Hua R, Laake P, Olofsson AM, Poblete-Naredo I, Rusakov DA, Madsen KK, Clausen RP, Schousboe A, White HS, Danbolt NC (2011) Deletion of the betaine-GABA transporter (BGT1; slc6a12) gene does not affect seizure thresholds of adult mice. Epilepsy Res 95:70-81.

Lehre KP, Danbolt NC (1998) The number of glutamate transporter subtype molecules at glutamatergic synapses: chemical and stereological quantification in young adult rat brain. J Neurosci 18:8751-8757.

Lehre KP, Levy LM, Ottersen OP, Storm-Mathisen J, Danbolt NC (1995) Differential expression of two glial glutamate transporters in the rat brain: quantitative and immunocytochemical observations. J Neurosci 15:1835- 1853.

Levy LM, Lehre KP, Rolstad B, Danbolt NC (1993) A monoclonal antibody raised against an [Na^+- K^+]coupled L- glutamate transporter purified from rat brain confirms glial cell localization. FEBS Lett 317:79- 84.

Lowry OH (1953) The quantitative histochemistry of the brain. J Histochem Cytochem 1:420-428.

Lowry OH, Roberts NR, Leiner KY, Wu ML, Farr AL, Albers RW (1954) The quantitative histochemistry of brain, III. ammon's horn. J Biol Chem 207:39-49.

Mathews GC, Diamond JS (2003) Neuronal glutamate uptake contributes to GABA synthesis and inhibitory synaptic strength. J Neurosci 23:2040-2048.

Otis TS, Kavanaugh MP, Jahr CE (1997) Postsynaptic glutamate transport at the climbing fiber Purkinje cell synapse. Science 277:1515- 1518.

Palay SL, Chan-Palay V (1974) Cerebellar Cortex - Cytology and Organization, pp 348. Berlin, Heidelberg, New York: PC Springer-Verlag.

Peghini P, Janzen J, Stoffel W (1997) Glutamate transporter EAAC-1- deficient mice develop dicarboxylic aminoaciduria and behavioral abnormalities but no neurodegeneration. EMBO J 16:3822-3832.

Peters A, Palay SL, Webster HF (1991) The Fine Structure of the Nervous System - Neurons and Their Supporting Cells, pp 512. New York, Oxford: Oxford University Press.

Pines G, Danbolt NC, Bjørås M, Zhang Y, Bendahan A, Eide L, Koepsell H, Storm-Mathisen J, Seeberg E, Kanner BI (1992) Cloning and expression of a rat brain L- glutamate transporter. Nature 360:464-467.

Plachez C, Danbolt NC, Recasens M (2000) Transient expression of the glial glutamate transporters GLAST and GLT in hippocampal neurons in primary culture. J Neurosci Res 59:587-593.

Ross JR, Porter BE, Buckley PT, Eberwine JH, Robinson MB (2011) mRNA for the EAAC1 subtype of glutamate transporter is present in neuronal dendrites in vitro and dramatically increases in vivo after a seizure. Neurochem Int 58:366-375.

Rothstein JD, Dykes-Hoberg M, Pardo CA, Bristol LA, Jin L, Kuncl RW, Kanai Y, Hediger MA, Wang YF, Schielke JP, Welty DF (1996) Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate. Neuron

16:675-686.
Rothstein JD, Martin L, Levey AI, Dykes-Hoberg M, Jin L, Wu D, Nash N, Kuncl RW (1994) Localization of neuronal and glial glutamate transporters. Neuron 13:713-725.
Scimemi A, Tian H, Diamond JS (2009) Neuronal transporters regulate glutamate clearance, NMDA receptor activation, and synaptic plasticity in the hippocampus. J Neurosci 29:14581-14595.
Sepkuty JP, Cohen AS, Eccles C, Rafiq A, Behar K, Ganel R, Coulter DA, Rothstein JD (2002) A neuronal glutamate transporter contributes to neurotransmitter GABA synthesis and epilepsy. J Neurosci 22:6372-6379.
Shashidharan P, Huntley GW, Murray JM, Buku A, Moran T, Walsh MJ, Morrison JH, Plaitakis A (1997) Immunohistochemical localization of the neuron-specific glutamate transporter EAAC1 (EAAT3) in rat brain and spinal cord revealed by a novel monoclonal antibody. Brain Res 773:139-148.
Sheldon AL, Gonzalez MI, Robinson MB (2006) A carboxyl- terminal determinant of the neuronal glutamate transporter, EAAC1, is required for platelet-derived growth factor-dependent trafficking. J Biol Chem 281:4876- 4886.
Stafford MM, Brown MN, Mishra P, Stanwood GD, Mathews GC (2010) Glutamate spillover augments GABA synthesis and release from axodendritic synapses in rat hippocampus. Hippocampus 20:134-144.
Storck T, Schulte S, Hofmann K, Stoffel W (1992) Structure, expression, and functional analysis of a Na^+-dependent glutamate/aspartate transporter from rat brain. Proc Natl Acad Sci U S A 89:10955-10959.
Sun W, Hoffman KM, Holley DC, Kavanaugh MP (2011) Specificity and Actions of an Arylaspartate Inhibitor of Glutamate Transport at the Schaffer Collateral-CA1 Pyramidal Cell Synapse. 6:e23765.
Takagaki G (1978) Sodium and potassium ions and accumulation of labelled D- aspartate and GABA in crude synaptosomal fraction from rat cerebral cortex. J Neurochem 30:47-56.
Tanaka K, Watase K, Manabe T, Yamada K, Watanabe M, Takahashi K, Iwama H, Nishikawa T, Ichihara N, Hori S, Takimoto M, Wada K (1997) Epilepsy and exacerbation of brain injury in mice lacking the glutamate transporter GLT- 1. Science 276:1699-1702.
Torp R, Hoover F, Danbolt NC, Storm-Mathisen J, Ottersen OP (1997) Differential distribution of the glutamate transporters GLT1 and rEAAC1 in rat cerebral cortex and thalamus: an in situ hybridization analysis. Anat Embryol (Berl) 195:317- 326.
Trotti D, Volterra A, Lehre KP, Rossi D, Gjesdal O, Racagni G, Danbolt NC (1995) Arachidonic acid inhibits a purified and reconstituted glutamate transporter directly from the water phase and not via the phospholipid membrane. J Biol Chem 270:9890-9895.
Ullensvang K, Lehre KP, Storm-Mathisen J, Danbolt NC (1997) Differential developmental expression of the two rat brain glutamate transporter proteins GLAST and GLT. Eur J Neurosci 9:1646-1655.
Weibel ER (1979) Stereological methods Vol. 1. Practical methods for biological morphometry. Academic press.
Woolley CS, McEwen BS (1992) Estradiol mediates fluctuation in hippocampal synapse density during the estrous cycle in the adult rat. J Neurosci 12:2549-2554.

V

VI