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Title: Neuronal vs glial glutamate uptake: resolving the conundrum

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Abstract
Neither normal brain function nor the pathological processes involved in neurological diseases can be adequately understood without knowledge of the release, uptake and metabolism of glutamate. The reason for this is that glutamate (a) is the most abundant amino acid in the brain, (b) is at the cross-roads between several metabolic pathways, and (c) serves as the major excitatory neurotransmitter. In fact most brain cells express glutamate receptors and are thereby influenced by extracellular glutamate. In agreement, brain cells have powerful uptake systems that constantly remove glutamate from the extracellular fluid and thereby limit receptor activation. It has been clear since the 1970s that astrocytes and neurons express glutamate transporters. The relative contribution of neuronal and glial transporters to the total glutamate uptake activity, however, as well as their functional importance, has been hotly debated ever since. The present short review provides (a) an overview of what we know about neuronal glutamate uptake as well as an historical description of how we got there, and (b) an hypothesis reconciling apparently contradicting observations thereby possibly resolving the paradox.

ABBREVIATIONS
Cre, Cyclization recombinase (Le and Sauer, 2000); EAAC1, rabbit glutamate transporter (EAAT3; slc1a1; Kanai and Hediger, 1992); EAAT, excitatory amino acid transporter (synonym to glutamate transporter); GABA, γ-aminobutyric acid; GLAST, rat glutamate transporter (EAAT1; slc1a3; Storck *et al.*, 1992; Tanaka, 1993a); GLT-1, rat glutamate transporter (EAAT2; slc1a2; Pines *et al.*, 1992); GLUL, glutamine synthetase; TBOA, DL-*threo*-*β*-benzyloxyaspartate

1. **Introduction**

It was noted early on that glutamate is a key player in brain metabolism (Krebs, 1935) and that brain cells have a remarkable ability to take up glutamate from the extracellular fluid (Stern *et al.*, 1949). Unexpectedly, glutamate was found to have an excitatory effect on neurons (Hayashi, 1954; Lucas and Newhouse, 1957; Curtis *et al.*, 1959; Curtis *et al.*, 1960) and investigators started to speculate whether glutamate might be a neurotransmitter (for review see: Roberts et al., 1981; Schousboe, 1981; Danbolt, 2001; Watkins and Evans, 1981; Fonnum, 1984; Erecinska and Silver, 1990; Broman *et al.*, 2000; McKenna, 2007; Hertz, 2013). As glutamate uptake turned out to be the only mechanism able to remove extracellular glutamate, it followed that the brain had to be dependent on glutamate uptake in order to control the excitatory action of glutamate (Logan and Snyder, 1971; Logan and Snyder, 1972; Wofsey *et al.*, 1971; Balcar and Johnston, 1972; Johnston, 1981). In agreement, glutamate uptake was shown to protect neurons in culture against glutamate (Frandsen and Schousboe, 1990; Rosenberg *et al.*, 1992). This realization lead to a strong interest in glutamate uptake as perturbations in glutamate uptake might cause or aggravate neurological disease. For instance, because the uptake process is driven by the transmembrane ion gradients of K^+ and Na^+(Kanner and Schuldiner, 1987; Nicholls and Attwell, 1990; Barbour *et al.*, 1991; Danbolt, 2001), it follows that a compromised brain energy supply (e.g. ischemia) will impair the transport process (for review see: Szatkowski and Attwell, 1994; Rossi *et al.*, 2000; Danbolt, 2001; Larsson *et al.*, 2004; Grewer and Rauen, 2005; Tzingounis and Wadiche, 2007). A full discussion about molecular properties and roles of glutamate transporters in disease, however, is beyond the scope of this review as that topic is covered elsewhere (for review see for example: Lipton and Rosenberg, 1994; Danbolt, 2001; Sattler and Rothstein, 2006; Beart and O'Shea, 2007; Sheldon and Robinson, 2007; Bröer and Palacin, 2011; Vandenberg and Ryan, 2013; Robert and Sontheimer, 2014; Robert *et al.*, 2014; Karki *et al.*, 2015; Soni *et al.*, 2014; Takahashi *et al.*, 2015).

Relevant to the present review is the fact that both glial cells (e.g. Henn *et al.*, 1974) and neurons (their axon-terminals in particular; Wofsey *et al.*, 1971; Beart, 1976) possess high-affinity glutamate uptake mechanisms. This was clear already in the 1970s, but the relative importance of the neuronal (axon-terminal) and the astroglial uptake systems has been debated ever since.

2. Early observations of neuronal glutamate uptake

Evidence for significant glutamate uptake in neurons came from several sources: **(a)** It was noted that neurons cultured alone are able to take up glutamate with high affinity (e.g. Drejer *et al.*, 1982; Waniewski and Martin, 1983; Sher and Hu, 1990; Frandsen and Schousboe, 1990; Balcar, 1991; Wang *et al.*, 1998a; Plachez *et al.*, 2004). **(b)** Axon-terminals are believed to reseal during homogenization of brain tissue and thereby form metabolically active structures. These pinched off nerve endings are called "synaptosomes" (Gray and Whittaker, 1962; Whittaker, 1969), and they can be used in uptake assays (Gfeller *et al.*, 1971; Logan and Snyder, 1972; Levi and Raiteri, 1973a; Levi and Raiteri, 1973b; Dodd *et al.*, 1981; Erecinska *et al.*, 1996; Robinson, 1998; Raiteri and Raiteri, 2000). Although it was understood that fragments of some of the smallest astrocyte branches can reseal like axon-terminals, and that this would contaminate the synaptosome preparations (e.g. Delaunoy *et al.*, 1979; Henn *et al.*,

1976; Nakamura *et al.*, 1993), it was believed that the resealing frequency of astrocytes was lower than that of axon-terminals. If so, then neuronal uptake would be relatively better preserved in the homogenates than that of other cell types. **(c)** Radiolabeled excitatory amino acids (L-[^{3}H]glutamate and D-[^{3}H]aspartate) accumulated preferentially in nerve terminals also when presented to brain slices *in vitro* (Beart, 1976; Storm-Mathisen and Iversen, 1979; Taxt and Storm-Mathisen, 1984), or infused *in vivo* at low concentrations and for short duration (Storm-Mathisen and Wold, 1981). While a predominance of neuronal over astroglial uptake in synaptosome preparations could be disregarded as a consequence of differences in cellular geometry and thereby in resealing frequency (see section 10 below), a predominance of neuronal uptake in hippocampal slices was harder to explain without assuming that axon-terminals had a considerable uptake activity. **(d)** Interruption of putative glutamatergic fibers caused a marked decrease in glutamate uptake activity in the target regions of these fibers (Fig. 1). The uptake activities in the target areas were determined using synaptosomes or brain slices (Divac *et al.*, 1977; McGeer *et al.*, 1977; Storm-Mathisen, 1977; Fonnum *et al.*, 1981; Taxt and Storm-Mathisen, 1984). Thus, destruction of neuronal cell bodies led to reduced glutamate uptake far away from the site of the lesion. Based on this it was assumed that the reductions in uptake activity were due to loss of nerve terminals rather than of astrocytes. **(e)** Selective retrograde labeling of neurons was observed after D-[^{3}H]aspartate microinjections or implantations further arguing that neuronal uptake was high relative to the astroglial uptake (Baughman and Gilbert, 1980; Streit, 1980; Fischer *et al.*, 1986).

These studies reinforced each other, and some investigators even thought that high-affinity glutamate uptake was the best marker for identification of glutamatergic nerve terminals (for review see: Fonnum, 1984; Ottersen and Storm-Mathisen, 1984). However, weaknesses in the arguments (see below) were highlighted after the glutamate transporters were cloned and localized.

3. Identification of plasma membrane glutamate transporters

The first glutamate transporter to be purified in active form (Danbolt *et al.*, 1990) was the one now known as EAAT2 (GLT-1; slc1a2; Pines *et al.*, 1992). The purification was based on solubilization of rat brain membranes with a detergent (cholate) followed by fractionation using conventional chromatographic techniques. Because the transporters could only be identified by their transport function, reconstitution of transport activity in artificial cell membranes (liposomes) was the only way to assay the various protein fractions for transporter content (Danbolt *et al.*, 1990). This approach resulted in a preparation of a glutamate transporter protein which was later confirmed to be pure although it was only enriched about hundred times (Levy *et al.*, 1993; Lehre and Danbolt, 1998). In fact, EAAT2 was found to represent 1 % of the total forebrain protein (Lehre and Danbolt, 1998). Antibodies were raised to the purified protein and used to localize it in the brain (Danbolt *et al.*, 1992; Levy *et al.*, 1993) and to screen expression libraries (Pines *et al.*, 1992). Simultaneously, but independently of each other, three other research teams succeeded in cloning another two glutamate transporters using completely different approaches: EAAT1 (GLAST; slc1a3; Storck *et al.*, 1992; Tanaka, 1993a) and EAAT3 (EAAC1; slc1a1; Kanai and Hediger, 1992; Kanai *et al.*, 1993; Bjørås *et al.*, 1996). The three human counterparts were quickly identified (Arriza *et al.*,

1994). Another two glutamate transporters were found later: EAAT4 (slc1a6; Fairman *et al.*, 1995) and EAAT5 (slc1a7; Arriza *et al.*, 1997). Thus, the mammalian genome contains five glutamate (or excitatory amino acid) transporter genes (for review see for instance: Danbolt, 2001; Beart and O'Shea, 2007; Tzingounis and Wadiche, 2007; Zhou and Danbolt, 2013; Vandenberg and Ryan, 2013; Grewer *et al.*, 2014; Fontana, 2015).

All the EAATs catalyze coupled transport of $1H^+$, $3Na^+$, and $1K^+$ with 1 substrate molecule (Klöckner *et al.*, 1993; Zerangue and Kavanaugh, 1996a; Levy *et al.*, 1998; Owe *et al.*, 2006). L-Glutamate and DL-aspartate are transported with similar affinities while D-glutamate is not. The transporters are actually complex molecules as they also function as chloride channels (Fairman *et al.*, 1995; Zerangue and Kavanaugh, 1996a, Wadiche *et al.*, 1995a; Wadiche *et al.*, 1995b; Wadiche and Kavanaugh, 1998; Bergles *et al.*, 2002; Koch *et al.*, 2007; Ryan and Mindell, 2007; Zhou *et al.*, 2014a; Fahlke *et al.*, 2016; LeVine *et al.*, 2016) and even transport water (MacAulay *et al.*, 2001, MacAulay *et al.*, 2004). Although the mammalian transporters have not yet been crystallized, we know quite a lot about their structure (Kanner, 2007; Vandenberg and Ryan, 2013; Gouaux, 2009; Verdon *et al.*, 2014; Silverstein *et al.*, 2015; Fahlke *et al.*, 2016; LeVine *et al.*, 2016).

The functional properties differ between the subtypes (Tzingounis and Wadiche, 2007; Vandenberg and Ryan, 2013). EAAT1-3 resemble each other in that they have relatively short cycling times and fairly similar affinities (Grewer and Rauen, 2005, Otis and Kavanaugh, 2000, Bergles *et al.*, 2002). The Km values reported for L-glutamate transport by EAAT1 are typically in the range 7-20 μM (Arriza *et al.*, 1994; Wadiche and Kavanaugh, 1998; Klöckner *et al.*, 1993). The corresponding values for EAAT2 and EAAT3 are, respectively, 12-18 μM (Arriza *et al.*, 1994; Levy *et al.*, 1998; Bergles *et al.*, 2002) and 8-30 μM (Arriza *et al.*, 1994; Kanai *et al.*, 1994; Kanai and Hediger, 1992; Grewer *et al.*, 2000). The kinetics of EAAT4 and EAAT5 are quite different (Mim *et al.*, 2005; Gameiro *et al.*, 2011). EAAT4 has the highest affinity with reported Km values in the range 0.6-3.3 μM (Fairman *et al.*, 1995; Lin *et al.*, 1998; #19512}; Mim *et al.*, 2005), while EAAT5 has the lowest affinity with Km values in the range 61-63 μM (Gameiro *et al.*, 2011; Schneider *et al.*, 2014).

4. Heterogeneity of glutamate uptake

A heterogeneity in the glutamate uptake system was expected early on based on differences between various cell cultures and tissue preparations with respect to substrate selectivity (Ferkany and Coyle, 1986; Robinson *et al.*, 1991; Robinson *et al.*, 1993; Fletcher and Johnston, 1991; Balcar and Li, 1992; Rauen *et al.*, 1992). A detailed account of the substrate selectivity of the individual transporter is beyond the scope of the present review. For more information, we recommend the outstanding review by Bridges and co-workers as an introduction (Bridges *et al.*, 1999) and more recent reviews for the latest updates (e.g. Vandenberg *et al.*, 1997; Jensen and Bräuner-Osborne, 2004; Shigeri *et al.*, 2004; Bridges and Esslinger, 2005; Shimamoto and Shigeri, 2006; Shimamoto, 2008; Sagot *et al.*, 2008; Jensen *et al.*, 2009). Here we will only point out (a) that the commonly used uptake inhibitor dihydrokainate (DHK; CAS 52497-36-6) blocks EAAT2 with high selectivity over the other EAATs (Arriza *et al.*, 1994; Bridges *et al.*, 1999), and (b) that DL-*threo*-*β*-benzyloxyaspartate (TBOA; CAS 205309-81-5) and its variants (e.g. PMB-TBOA and TFB-TBOA) block all five of the

EAATs (Bridges *et al.*, 1999; Lebrun *et al.*, 1997; Shimamoto *et al.*, 1998; Shimamoto, 2008). These compounds are competitive inhibitors in the sense that they bind to the same binding sites as glutamate thereby competing with glutamate for binding to the transporter protein. Importantly, although DHK and TBOA can bind to the transporter like glutamate, they are too bulky to be moved through the transporter to the other side of the plasma membrane. In contrast, several other glutamate uptake inhibitors, e.g. D-aspartate, *threo*-3-hydroxyaspartate (CAS 7298-99-9; Balcar *et al.*, 1977) and L-*trans*-pyrrolidine-2,4-dicarboxylate (CAS 64769-66-0; Bridges *et al.*, 1991) are themselves transported. This means that they inhibit uptake of glutamate by being transported instead of glutamate, but, importantly, they are also exchanged (see Section 12 below) with internal glutamate and thereby induce glutamate release. Thus, when transportable uptake inhibitors are added to cell cultures, the extracellular glutamate concentration rises both because the extracellular glutamate is not removed and because more glutamate is coming out of the cells (Volterra *et al.*, 1996; O'Shea *et al.*, 2002).

5. Cellular distribution of glutamate transporters

Early studies on the distribution of glutamate uptake sites were based on autoradiography of radioactively labeled substrates or on the use of antibodies to substrates immobilized in the tissue by means of glutaraldehyde fixation (for review see: Zhou and Danbolt, 2014). After the transporters had been cloned and the sequences were known, transporter mRNA could be detected using *in-situ* hybridization (Harrison and Pearson, 1990; Berger and Hediger, 1998). Further, peptides representing parts of the transporter proteins could be synthesized and used to generate antibodies to the transporters themselves (Lehre *et al.*, 1995; Danbolt *et al.*, 1998) rather than to the substrates. Antibodies became readily available and a large number of papers were published. Unfortunately, not all antibodies and procedures were validated well enough (for discussion see: Holmseth *et al.*, 2005; Holmseth *et al.*, 2006; Rhodes and Trimmer, 2006; Lorincz and Nusser, 2008; Couchman, 2009; Kalyuzhny, 2009; Holmseth *et al.*, 2012a; Zhou *et al.*, 2014b). Further, rapid post mortem proteolysis of the termini of EAAT1 and EAAT2 represents an additional challenge when studying human samples (Beckstrøm *et al.*, 1999; Tessler *et al.*, 1999; Li *et al.*, 2012). Most of the immunoreactivity to the C-terminus of EAAT2 is gone after 24 hours and there is a significant reduction after 12 hours. The reduction is, however, not uniform, but happens apparently suddenly in each cell leaving entire astrocyte domains unlabeled. Further, EAAT1 is lost at a slower rate. At 12 hours a patchy image appears with many astrocytes not labeled for any of the transporters and some only positive for EAAT1. A very different pattern is seen with antibodies to the central parts of the EAAT2 protein which is much more stable (Li *et al.*, 2012). Consequently, the literature on transporter distributions is complex.

5.1. EAAT2 (GLT-1; slc1a2) was the first glutamate transporter to be localized immunocytochemically (Danbolt *et al.*, 1992; Lehre *et al.*, 1993; Levy *et al.*, 1993; Lehre *et al.*, 1995). The astrocytes were intensely labeled, while the neurons appeared unlabeled. Labeling was not even detected in glutamatergic axon-terminals in the stratum radiatum of the CA1 hippocampus (Danbolt *et al.*, 1992; Lehre *et al.*, 1995) despite high levels of EAAT2 mRNA in CA3 pyramidal cells from which they originate

(Torp *et al.*, 1994; Schmitt *et al.*, 1996; Torp *et al.*, 1997). Subsequent immunocytochemical studies confirmed this conclusion (e.g. Rothstein *et al.*, 1994; Schmitt *et al.*, 1996; Milton *et al.*, 1997; Kugler and Schmitt, 2003; Berger *et al.*, 2005; Holmseth *et al.*, 2009), while *in-situ* hybridization studies added that EAAT2 mRNA was also present in several neuronal populations (Berger and Hediger, 1998; Berger and Hediger, 2000; Berger and Hediger, 2001). Data obtained with EAAT2 eGFP BAC reporter mice (De Vivo *et al.*, 2010a) showed that the EAAT2 promotor is highly active in virtually all astrocytes (defined as cells expressing glial fibrillary acidic protein, GFAP), but also in several neuronal populations (including 80 % of the CA3 pyramidal cells), albeit at considerably lower levels than in astrocytes. This is in fairly good agreement with the in situ hybridization data (e.g. Berger and Hediger, 1998; Berger and Hediger, 2000; Berger and Hediger, 2001).

The failure to detect EAAT2 protein in neurons in the mature and normal brain was puzzling as both EAAT2 protein and mRNA were detected in several types of cultured neurons (e.g. Brooks-Kayal *et al.*, 1998; Mennerick *et al.*, 1998; Meaney *et al.*, 1998; Wang *et al.*, 1998a; Plachez *et al.*, 2000). Further, EAAT2 had also been reported in neurons in the normal and mature mammalian retina (Rauen, 2000; Harada *et al.*, 1998), transiently in growing axons of the mouse spinal cord before establishing astrocytic expression (Yamada *et al.*, 1998), and in brain neurons after hypoxia-ischemia (Martin *et al.*, 1997). Although it is to be expected that the behavior of cells in the normal and mature brain may differ from that of cells in culture or in diseased tissue (Cahoy *et al.*, 2008), this did not explain why many neurons in the mature brain express EAAT2 mRNA. Why couldn't the EAAT2 protein be detected? Could it be that EAAT2 mRNA had additional functions? After all, the length of the mRNA molecule was 11.3 kb or 6.6 times longer than the coding sequence (Pines *et al.*, 1992).

Adding to the puzzle, immunoprecipitation experiments showed that antibodies to EAAT2 could remove about 95 % of the total reconstitutable glutamate uptake activity in detergent (cholate) extracts from the normal young adult rat brain (Danbolt *et al.*, 1992; Haugeto *et al.*, 1996). In agreement, deletion of the EAAT2 gene in mice caused an almost complete loss (about 95 %) of glutamate uptake activity (Tanaka *et al.*, 1997; Otis and Kavanaugh, 2000; Matsugami *et al.*, 2006; Kiryk *et al.*, 2008; Holmseth *et al.*, 2012b; Zhou *et al.*, 2014b).

Homozygote EAAT2-knockout mice (Tanaka *et al.*, 1997) are inconspicuous at birth because EAAT2 expression is very low in newborn rats and mice (Ullensvang *et al.*, 1997; Furuta *et al.*, 1997; Hanson *et al.*, 2015), but they become spontaneously epileptic at around three weeks of age and about half of them have died by the end of the fourth week (Tanaka *et al.*, 1997; Mitani and Tanaka, 2003; Takasaki *et al.*, 2008). A selective deletion of EAAT2 in the brain is sufficient to reproduce this phenotype confirming that EAAT2 plays its most important role in the brain (Zhou *et al.*, 2014b). The heterozygote EAAT2 knockout mice (+/-) have only half the EAAT2-concentrations as wildtype mice, but do not show any apparent morphological brain abnormalities (Kiryk *et al.*, 2008). However, they are more vulnerable to traumatic spinal cord injury (Lepore *et al.*, 2011).

The high total expression levels of glutamate transporters (Bergles and Jahr, 1997; Dehnes *et al.*, 1998; Lehre and Danbolt, 1998; Otis and Kavanaugh, 2000) make sense when taking into account that low resting levels of extracellular glutamate are

obtained (Herman and Jahr, 2007) despite the slowness of the transport process (Otis and Jahr, 1998; Otis and Kavanaugh, 2000; Bergles *et al.*, 2002; Grewer and Rauen, 2005) and the rapid extracellular turnover of glutamate (Jabaudon *et al.*, 1999). The average number of EAAT-type of protein molecules is more than ten thousand per synapse (Table 1).

Given that EAAT2 was purified by monitoring uptake activity (Danbolt *et al.*, 1990), it was not surprising that EAAT2 is the major isoform, but such a dominant role was unexpected. Further, it was also hard to explain why the synaptosomal uptake activity was absent in the EAAT2 knockout mice (Tanaka *et al.*, 1997) considering that the protein had only been detected in astrocytes. This needed an explanation (see below).

5.2. EAAT1 (Storck *et al.*, 1992; Tanaka, 1993b; Arriza *et al.*, 1994) is the only glutamate transporter that appears to be selectively expressed in astroglial cells in the central nervous system (Danbolt, 1994; Lehre *et al.*, 1993; Lehre *et al.*, 1995; Ginsberg *et al.*, 1995; Rothstein *et al.*, 1995; Schmitt *et al.*, 1997; Ullensvang *et al.*, 1997; Berger and Hediger, 1998; Banner *et al.*, 2002; Kugler and Beyer, 2003; Regan *et al.*, 2007; Berger and Hediger, 2000; Jungblut *et al.*, 2012) including retina (Rauen *et al.*, 1996; Lehre *et al.*, 1997; Rauen *et al.*, 1998; Harada *et al.*, 1998; Pow and Barnett, 1999; Rauen, 2000; Bringmann *et al.*, 2013). It is also expressed in supporting cells in the inner ear (Furness and Lehre, 1997; Takumi *et al.*, 1997) as well as in several peripheral organs including, the heart, fat cells and taste buds (Lawton *et al.*, 2000; Berger and Hediger, 2006; Adachi *et al.*, 2007; Martinov *et al.*, 2014). Mice lacking EAAT1 (Watase *et al.*, 1998) have an increased prenatal lethality, but appear to develop normally after birth. They show, however, symptoms of insufficient glutamate uptake in regions where EAAT1 is the major glutamate transporter, e.g. cerebellum, inner ear and retina (Watase *et al.*, 1998; Hakuba *et al.*, 2000; Harada *et al.*, 1998; for more references see: Zhou and Danbolt, 2014). Lack of EAAT1 is thereby less dramatic than lack of EAAT2 (see above), but as the expression is not neuronal it will not be discussed further in this review.

5.3. EAAT3 (Kanai and Hediger, 1992; Arriza *et al.*, 1994; Bjørås *et al.*, 1996) is a neuronal transporter as originally suggested and it is not expressed in glial cells (Rothstein *et al.*, 1994; Shashidharan *et al.*, 1997; Holmseth *et al.*, 2012b). It appears to be expressed in most, if not all, neurons throughout the CNS. However, EAAT3 is not found in axon-terminals, and is selectively targeted to somata and dendrites (Shashidharan *et al.*, 1997; Cheng *et al.*, 2002; Holmseth *et al.*, 2012b). Within the CNS, EAAT3 is found in the highest concentrations in the hippocampus followed by the neocortex, but the total tissue content in young adult rat brains is about 100 times lower than that of EAAT2 (Holmseth *et al.*, 2012b).

Mice lacking EAAT3 (Peghini *et al.*, 1997) develop dicarboxylic aminoaciduria and have been reported to age prematurely (Aoyama *et al.*, 2006) possibly because EAAT3 transports cysteine (Zerangue and Kavanaugh, 1996b) which is needed for glutathione synthesis. However, EAAT3-deficient mice do not show signs of neurodegeneration at a young age and do not have epilepsy (Peghini *et al.*, 1997; Aoyama *et al.*, 2006; Berman *et al.*, 2011) in agreement with the low expression levels

compared to EAAT2. Humans lacking EAAT3 develop dicarboxylic aminoaciduria in agreement with the studies on mice (Bailey *et al.*, 2011), and human EAAT3 polymorphisms have been reported to be associated with obsessive–compulsive disorders (Brandl *et al.*, 2012; Walitza *et al.*, 2010; Grunblatt *et al.*, 2014).

EAAT3 has been particularly difficult to localize and, there are conflicting reports in the literature. The difficulties are in part due to the lower expression levels (Zhou and Danbolt, 2014), but mostly to the difficulties involved in obtaining specific antibodies (for discussions see: Danbolt *et al.*, 1998; Holmseth *et al.*, 2005; Holmseth *et al.*, 2006). It was not possible to distinguish between specific labeling of EAAT3 and cross-reactivity until tissue from the EAAT3-deficient mice became available (Peghini *et al.*, 1997). The so-called pre-adsorption test to validate antibody specificity did not help (Holmseth *et al.*, 2012a).

5.4. EAAT4 and **EAAT5** are, as already mentioned above, quite different from the other EAATs. They transport slowly and have very high chloride conductances (Mim *et al.*, 2005; Gameiro *et al.*, 2011).

EAAT4 (Fairman *et al.*, 1995) is expressed at fairly high levels in the dendrites and cell bodies belonging to the cerebellar Purkinje cells as well as in some forebrain neurons, but not in non-neuronal cells (Dehnes *et al.*, 1998; Massie *et al.*, 2008). This distribution pattern has been confirmed by using EAAT4 promotor reporter mice (Gincel *et al.*, 2007; De Vivo *et al.*, 2010b). There is also EAAT4 in vestibular hair cells and calyx endings (Dalet *et al.*, 2012). EAAT4 is thereby considered to be neuron selective (apart from expression in vestibular hair cells), but it does not appear to be of critical importance as EAAT4 knockout mice are viable and appear normal (Huang *et al.*, 2004) albeit with some alteration of receptor activation (Nikkuni *et al.*, 2007). Although EAAT4 in Purkinje cells removes more glutamate than EAAT3, EAAT4 is removing less than 10% of the glutamate released by the climbing fibers (Huang *et al.*, 2004).

EAAT5 (Arriza *et al.*, 1997) is present in the retina at significant levels (Eliasof *et al.*, 1998; Veruki *et al.*, 2006; Schneider *et al.*, 2014), and it may be subject to variable splicing (Lee *et al.*, 2012), but has not been reported in other parts of the central nervous system. It is present in vestibular hair cells (Dalet *et al.*, 2012), in the heart (Arriza *et al.*, 1997; Martinov *et al.*, 2014), in skeletal muscle (Arriza *et al.*, 1997) and possibly at low levels in several other peripheral organs (Lee *et al.*, 2013).

6. Weaknesses in the old evidence for neuronal glutamate uptake

When the first immunocytochemical data appeared on the distribution of the various glutamate transporters, it became clear the transporter in glutamatergic nerve terminals had not been identified (Danbolt, 2001). Of course, a novel transporter with similar properties might still remain undiscovered, but EAAT2 could (as explained in Section 5.1 above) account for almost all of the uptake activity giving little room for hypothetical undiscovered glutamate transporters: (a) Antibodies to the glutamate transporter subtype EAAT2 could immunoprecipitate most of the reconstitutable glutamate uptake activity present in brain extracts (see Section 5.1 for references). (b) Synaptosomes prepared from EAAT2-deficient mutant mice had very low uptake activities (see Section 5.1 for references). (c) Lesioning of glutamatergic pathways resulted in down-regulation of glial glutamate transporters in the target region, and thereby had effects that were not

limited a simple loss of axon-terminals (Fig. 1; Levy *et al.*, 1995; Ginsberg *et al.*, 1995; Rao *et al.*, 1998). This observation indicated that glial expression of glutamate transporters depends, at least in part, on soluble factors (Fig. 2; Gegelashvili *et al.*, 1997; Gegelashvili *et al.*, 2000; Plachez *et al.*, 2000; Martinez-Lozada *et al.*, 2016). In other words, investigators asked if the reductions in synaptosomal uptake observed in the target areas of lesioned fibers could be attributed to a loss of glial uptake sites. (d) The glutamate uptake in neuronal cultures (Wang *et al.*, 1998b) and in synaptosome preparations was shown to be sensitive to inhibition by kainate and dihydrokainate (Johnston *et al.*, 1979; Robinson *et al.*, 1993; Bridges *et al.*, 1999) just like EAAT2 which is the only glutamate transporter that is sensitive to inhibition by these compounds (Arriza *et al.*, 1994; Martinez-Lozada *et al.*, 2016). (e) Also the autoradiographic studies aimed at localizing the glutamate uptake sites in hippocampal slices could be criticized because they were either (Beart, 1976; Storm-Mathisen and Iversen, 1979) done at the electron microscopical level with [^{3}H]glutamate (which may be converted to [^{3}H]glutamine in glial cells and then back to [^{3}H]glutamate in the terminals or (Taxt and Storm-Mathisen, 1984) with D-[^{3}H]aspartate at the light microscopical level where labeling of terminals cannot be rigorously distinguished from labeling of surrounding fine glial processes.

Based on the above, there was good reason to question (Bridges *et al.*, 1999; Koch *et al.*, 1999) if the synaptosomal uptake is due to uptake in glial cell fragments contaminating these preparations (Henn *et al.*, 1976; Nakamura *et al.*, 1993) rather than to uptake in the synaptosomes (nerve terminals) themselves.

7. Axon-terminals take up glutamate by means of EAAT2

As explained above (see Section 6), the very existence of a high affinity glutamate uptake mechanism in nerve terminals was questioned. To address the question directly avoiding the above weaknesses, it was decided to develop antibodies specifically recognizing glutaraldehyde-fixed D-aspartate (Zhang *et al.*, 1993). These antibodies reacted strongly with D-aspartate without cross-reacting with other relevant amino acids like L-aspartate, glutamine and L-glutamate (Zhang *et al.*, 1993; Gundersen *et al.*, 1993) or with aldehyde fixed brain tissue in general. D-aspartate was chosen because **(a)** it is a substrate for the EAAT-type of transporters; **(b)** there is hardly any D-aspartate present endogenously; **(c)** exogenously added D-aspartate is slowly metabolized in the adult brain (Davies and Johnston, 1976; Takagaki, 1978). Thus, detection of sodium-dependent high affinity uptake of D-aspartate in neurons would be proof of direct uptake rather than indirect uptake via astrocytes (c.f. glutamate-glutamine cycle).

Hippocampal slices were incubated with D-aspartate, fixed with glutaraldehyde and processed for immunogold-labeling with the anti-D-aspartate antibodies. By electron microscopy it could be shown that D-aspartate accumulated in a sodium dependent manner in glutamatergic terminals in rat stratum radiatum in hippocampus CA1 (Gundersen *et al.*, 1993) and in rat striatum (Gundersen *et al.*, 1996). This implied that terminals did have the ability to take up glutamate (Fig. 3), but the identity of the transporter responsible was still unknown. After the discovery that dihydrokainate is a selective inhibitor of EAAT2 (Arriza *et al.*, 1994), it was decided to repeat the slice experiments. Then it turned out that the accumulation of D-aspartate in the terminals could be blocked by adding dihydrokainate together with D-aspartate (Furness *et al.*,

2008). Further, using hippocampal slices from the EAAT2-knockout mice (Tanaka *et al.*, 1997) it became clear that there was no D-aspartate uptake in axon-terminals in CA1 in mice lacking the EAAT2 gene (Furness *et al.*, 2008).

We also examined D-aspartate uptake in hippocampal synaptosome preparations in the same way (Furness *et al.*, 2008). About 70 % of the nerve terminals in these preparations were D-aspartate positive, and they represented 85 % of the D-aspartate immunoreactivity implying that most of the uptake was due to terminals.

These experiments showed that axon-terminals accumulate D-aspartate by a sodium-dependent mechanism that is sensitive to inhibition by dihydrokainate and that depends on the EAAT2-gene. This strongly suggested that EAAT2 is the glutamate transporter in nerve terminals. However, one important piece of evidence was still missing: EAAT2 protein had not been detected in the terminals.

8. EAAT2 protein in axon-terminals

Could EAAT2 be expressed in axon-terminals and somehow have evaded detection? One possibility could be splice variants (Lauriat and Mcinnes, 2007) not recognized by the antibodies, although the available array of anti-EAAT2 antibodies recognizing different parts of the EAAT2 molecule made that somewhat unlikely (see: Holmseth *et al.*, 2005; Holmseth *et al.*, 2009). Nevertheless, this was a possibility considering that there are multiple EAAT2 splice variants (e.g. Meyer *et al.*, 1998a; Meyer *et al.*, 1998b; Meyer *et al.*, 1998c; Honig *et al.*, 2000; Flowers *et al.*, 2001; Berger *et al.*, 2005; Sullivan *et al.*, 2004; Rauen *et al.*, 2004; Peacey *et al.*, 2009). One of these variants might be selective for the axon-terminals. In fact, it was first thought that EAAT2b (GLT-1b) was the neuronal transporter, but it was later realized that the antibodies had not been good enough, and that EAAT2a might be the one in terminals (Chen *et al.*, 2004). It is now recognized that both EAAT2a and EAAT2b are predominantly astroglial (Berger *et al.*, 2005; Holmseth *et al.*, 2009), and that the total tissue content of EAAT2a protein is one order of magnitude higher than that of EAAT2b (Holmseth *et al.*, 2009). Thus, EAAT2a is the predominant variant both in astroglia and in axon-terminals (Furness *et al.*, 2008; Holmseth *et al.*, 2009) in agreement with Chen and co-workers (2004).

The study by Chen and co-workers (2004) was based on pre-embedding electron microscopy and was not quantitative beyond determination of the number cell profiles with labeling significantly above background (for a description of these methods see: Danbolt *et al.*, 1998; Amiry-Moghaddam and Ottersen, 2013). It was therefore important to do post-embedding immuno-gold which permits comparisons of labeling intensities. A limitation with the latter technique, however, is that the gold-particles attached to antibodies can swing around and thereby be located quite some distance from the antigen. Therefore if plasma membranes from two different cells are too close to each other, then it may not be possible to use immuno-gold to determine which cell the antigen belongs to (Danbolt *et al.*, 1998; D'Amico and Skarmoutsou, 2008; Amiry-Moghaddam and Ottersen, 2013). Fortunately, the hippocampal slice preparations used (Furness *et al.*, 2008) had an advantage over perfusion fixed tissue (Danbolt *et al.*, 1998): namely that the extracellular spaces were larger and, interestingly, quite similar to tissue prepared by high-pressure freezing techniques (Korogod *et al.*, 2015). Thus, the distance between neighboring plasma membranes was greater and frequently

further than the mobility of the gold-particles. This was very helpful because it became easier to interpret the immuno-gold labeling. About 80 % of the gold-particles were found to be associated with astroglia. The rest was distributed over other structures. About 6 % was in nerve terminal membranes, while most the remainder was distributed over small axons (Furness *et al.*, 2008). These data are from the young adult rat stratum radiatum hippocampus CA1. Interestingly, the total amounts of plasma membranes in the stratum radiatum per cubic micrometer of tissue was 14 square micrometer of which about 10 % belonged to astrocytes, about 10 % to dendritic spines and about 10 % to terminals, while most of the remainder was due to small axons (Lehre and Danbolt, 1998; Furness *et al.*, 2008; Holmseth *et al.*, 2012b). Thus, the EAAT2 density over terminals was about 10 % of that over astroglia. This is still about ten times higher than the density of EAAT3 in dendritic spines and dendrites (Holmseth *et al.*, 2012b). As mentioned above, EAAT3 is absent from axon-terminals.

Because the glutamatergic terminals in the stratum radiatum CA1 originate mostly from the CA3 pyramidal cells, it makes sense that the latter cells have high levels of EAAT2 mRNA in agreement with recent observation from EAAT2 eGFP BAC reporter mice (De Vivo *et al.*, 2010a) as well as with in-situ hybridization studies (for references, see above).

9. Astroglial EAAT2 is the major protector against excitotoxicity

As explained above, EAAT2 is expressed both in astrocytes and in axon-terminals, and there is no available method to distinguish between neuronal and astroglial EAAT2 pharmacologically. Further, in the *conventional* (or *global*) EAAT2-knockout mice (Tanaka *et al.*, 1997) the EAAT2 gene was deleted from conception onwards in all cells (thus in both neurons and glia as well as in peripheral organs). Therefore, the obvious thing to do was to create *conditional (cell-type selective)* knockouts by using the Cre-LoxP recombinase system (Gu *et al.*, 1993; Rajewsky *et al.*, 1996; Le and Sauer, 2000). The EAAT2 gene was tagged by inserting a unique unidirectional DNA sequence called a "LoxP-site" before and after the stretch of DNA to be deleted. Genes with LoxP sites on each side are referred to as being "floxed" (an acronym for "flanking LoxP sites"). Cell selective deletion of the EAAT2 gene can then be achieved by controlling the expression of Cyclization recombinase (Cre). This is an enzyme that originates from Enterobacteria phage P1, binds to two LoxP-sites and recombines the DNA leaving only one loxP after deleting the sequence in between. The most common way of introducing expression of Cre is to cross flox-mice with Cre-mice (mice harboring the Cre gene). It is important to keep in mind that if Cre protein has been expressed at one point in time, then irreversible deletion is likely to have occurred implying that the gene is gone from the Cre-expressing cells as well as from all of their descendants.

Three different research teams generated, independently of each other, their own flox-EAAT2 mice (Zhou *et al.*, 2014b; Petr *et al.*, 2015; Aida *et al.*, 2015). The first of these three mouse lines (Zhou *et al.*, 2014b) is already available from Jackson Laboratory (JAX Stock No. 026619).

Nestin11-Cre mice (Tronche *et al.*, 1999; Cat. no. 003771; The Jackson Laboratory, Bar Harbor, Maine, USA) express Cre in all of the cells that give rise to the nervous system, but neither in the liver nor in the endocrine pancreas. Thus, crossing Nestin11-Cre mice with flox-EAAT2 mice resulted in mice lacking EAAT2 in the brain

(Zhou *et al.*, 2014b). These mice developed spontaneous epilepsy and died early similarly to the conventional knockout mice (Tanaka *et al.*, 1997). In contrast, crossing with pancreas selective Cre-lines had apparently no consequences. This confirmed that EAAT2 plays its main roles in the nervous system and a role in pancreatic insulin secretion was ruled out (Zhou *et al.*, 2014b).

The next step was to delete EAAT2 selectively in astrocytes. This, however, was not straight forward. Although GFAP is an astrocyte marker in the adult brain and GFAP-Cre lines allow deletion in astrocytes (e.g. Marino *et al.*, 2000; Zhuo *et al.*, 2001), these lines are not fully specific for astrocytes because the GFAP promotor is active also in radial glia which gives rise to virtually all neocortical projection neurons (Malatesta *et al.*, 2003). Therefore a *tamoxifen inducible* human glial fibrillary acidic protein hGFAP-CreERT2 (Casper *et al.*, 2007) mouse line was employed. These mice only express Cre when both the GFAP promotor is active and when tamoxifen is administered. Thus, Cre-mediated expression in neurons can be avoided by injecting tamoxifen at postnatal day 5 or later. This resulted in mice where the levels of EAAT2 protein in the whole forebrain were reduced to 1/5 of the normal level resulting in spontaneous epilepsy and increased mortality (Petr *et al.*, 2015) albeit not quite as dramatic as the global knockouts. In contrast, deletion in neurons by crossing the flox-EAAT2 mice with synapsin I promoter-driven Cre recombinase mice (Rempe *et al.*, 2006; Jackson Laboratory: JAX Stock No. 003966) barely affected the total brain EAAT2 protein levels. The mice appeared normal with normal lifespan. From this it was concluded that astroglial EAAT2 is the main protector against excitotoxicity (Petr *et al.*, 2015) in agreement with the distribution of the EAAT2 protein.

10. Resealing probability favors neuronal EAAT2 in synaptosome preparations

When brain tissue is homogenized, long cellular extensions will rupture. Nerve terminals (boutons) are structures with diameters typically in the range 250 - 600 nm (Sorra and Harris, 1998; Ventura and Harris, 1999; Furness *et al.*, 2008), and which are connected via axons that have diameters considerably smaller than the diameter of the terminals. When an axon is pulled off, it is therefore easy to imagine that many of the terminals may be able to reseal and thereby preserve their transmembrane ion gradients (Gray and Whittaker, 1962; Whittaker, 1969; Dodd *et al.*, 1981). In contrast, when astrocyte extensions are ruptured and astrocyte fragments created, then the diameter of the rupture site on each fragment will often be larger than the diameter of the fragment. This makes it plausible that the percentage of astrocyte fragments that reseal successfully will be considerably lower than the percentage of resealing terminals. And as long as a fragment is leaky, there can neither be any ion gradients to drive the transport nor any containment of the transported molecules. Thus, such fragments will not contribute to the measured uptake.

As explained above, while the density of EAAT2 molecules is about 10 times higher on astrocytes than on terminals (in stratum radiatum, hippocampus CA1), the plasma membrane surface areas are about the same (Furness *et al.*, 2008). Thus, if the resealing probability is lower, then the 10 % of EAAT2 in terminals may contribute significantly to the uptake activity measured in a homogenate. In agreement, it was shown electron microscopically that D-aspartate accumulates preferentially (85 %) in the terminals in synaptosome preparations (Furness *et al.*, 2008). This was tested using

conditional knockout mice: when EAAT2 was selectively deleted in neurons (using synapsin-Cre mice), then the uptake activity in synaptosome preparations was reduced to half (Petr *et al.*, 2015; see also Fig. 4). In contrast, when EAAT2 was deleted in astrocytes, then there was only a minor reduction in synaptosomal uptake activity (Petr *et al.*, 2015). As explained above, the most likely reason for this discrepancy between the distribution of EAAT2 protein molecules (mostly in astrocytes) and uptake activity (mostly in terminals) was that most of the astrocytic EAAT2 protein was sitting in membrane fragments that had not resealed. To test this, brain tissue was solubilized and reconstituted in liposomes as described previously (Danbolt *et al.*, 1990; Trotti *et al.*, 1995). In this case all the transporters were taken out of their natural membranes (irrespective of whether they were in leaky or in tight compartments) and inserted into new membranes. Thus, all EAAT2 protein molecules would now have the same probability of ending up in closed membrane bounded structures. As expected, the uptake activity determined in the liposome assay correlated with the immunoblots. Thus, upon reconstitution, the quantitative dominance of the astroglial EAAT2 was revealed (Petr *et al.*, 2015).

A weakness with these experiments based on conditional knockout mice (Petr *et al.*, 2015) is that the synapsin-Cre mice do not appear to express Cre in all neurons. This implies that the reduction observed is likely to be an underestimation (Y. Zhou and N.C. Danbolt, manuscript in preparation) and may explain why deletion is neurons using synapsin-Cre resulted in a lower reduction of synaptosomal uptake (about half) than expected from the D-aspartate uptake experiments described above (85 %; Furness *et al.*, 2008; Section 7).

Nevertheless, these experiments showed that the synaptosome assay mostly detects neuronal components as originally assumed (Gfeller *et al.*, 1971; Logan and Snyder, 1972; Levi and Raiteri, 1973a; Levi and Raiteri, 1973b; Fonnum, 1984; Erecinska *et al.*, 1996; Robinson, 1998; Raiteri and Raiteri, 2000) despite contamination from astrocytes (e.g. Delaunoy *et al.*, 1979; Henn *et al.*, 1976; Nakamura *et al.*, 1993). Further, these data do not give any indications that nerve terminal EAAT2 is more active than astroglial EAAT2 (Petr *et al.*, 2015).

11. Mismatch between EAAT2 distribution and sites of D-aspartate accumulation

That nerve terminal uptake of glutamate is favored over astroglial uptake in tissue homogenates may, as explained in Section 10 above, simply be that the damage to plasma membranes during homogenization differentially affects nerve terminals and glia. The validity of this argument, however, is not quite as obvious in 0.3 mm thick hippocampal slices because glial cells in slices maintain large negative resting potentials, and their extensive gap junctional coupling should help to maintain the membrane potential and intracellular ion concentrations near normal values even if one particular cell is somewhat damaged (Bergles and Jahr, 1997). Consequently, it was a surprise to find that about half of all D-aspartate taken up by hippocampus slices occurs in axon-terminals considering that terminals only contain around 10 % of the EAAT2 protein (Furness *et al.*, 2008). One possibility could be that heteroexchange is faster than net uptake, but we have ruled that out as explained below (Section 12). Another possibility (that remains to be experimentally tested) might be loss from astrocytes as explained below (Section 13).

12. Heteroexchange is not substantially faster than net uptake

Could the disproportionately efficient uptake into terminals in hippocampal slices (Furness *et al.*, 2008) be due to heteroexchange being faster than net uptake? The astrocytes in the hippocampal slice preparations are believed to mostly perform net uptake because they maintain their transmembrane gradients (Bergles and Jahr, 1997) and because the internal glutamate levels are relatively low. This would favor net uptake which is a process whereby extracellular sodium ions and glutamate are brought into the cell in exchange for potassium. In contrast, we know little about the metabolic states of the terminals. They have much more internal glutamate and if their ion gradients should be weakened, then they might switch to heteroexchange mode.

How much glutamate is there in terminals compared to astrocytes? Although terminals are generally believed to have much higher glutamate levels than astrocytes, there is, as far as we are aware of, no comprehensive overview of the glutamate concentrations in the various cellular compartments in different brain regions of wildtype animals. It is, however, possible to do some very rough estimates by combining histology data from normal rats (Nafstad and Blackstad, 1966; Lehre and Danbolt, 1998) with available information on glutamate distribution (Ottersen, 1989; Storm-Mathisen and Ottersen, 1990; Torp *et al.*, 1991; Storm-Mathisen *et al.*, 1992; Ottersen *et al.*, 1992; Ottersen *et al.*, 1996). Based on this studies is seems reasonable to conclude that around 10 % of tissue glutamate is in astroglia, 70 % in glutamatergic nerve terminals and the remainder in other parts of the neurons.

Heteroexchange is a process whereby the transporters exchange external and internal substrate molecules in a 1:1 relationship (Levi and Raiteri, 1974; Raiteri *et al.*, 1975; Pines and Kanner, 1990; Zerangue and Kavanaugh, 1996a; Kavanaugh *et al.*, 1997; Zhou *et al.*, 2014a; see figure 5 in Danbolt, 2001). This process is electroneutral and has therefore been tricky to study with electrophysiological techniques. Further, it took time before it was understood how it could complicate the interpretations of binding assays (Pin *et al.*, 1984; Danbolt and Storm-Mathisen, 1986a; Danbolt and Storm-Mathisen, 1986b; Danbolt, 1994), and cell culture experiments (Volterra *et al.*, 1996; O'Shea *et al.*, 2002).

Heteroexchange does not contribute to glutamate clearance as there is no net transport, but it was argued that it might still explain the disproportionately strong labeling of glutamatergic terminals with D-aspartate. Thus, if externally added D-aspartate is exchanged with internal glutamate, D-aspartate would accumulate inside even though the total number of substrate molecules (D-aspartate + L-glutamate) on either side of the membrane is unchanged. Old data based on synaptosomes indicated that heteroexchange is ten times faster than net uptake (Erecinska and Nelson, 1987; for review, see: Grewer and Rauen, 2005). There was, however, no consensus as some electrophysiological measurements of the transporter cycling rate using the glutamate transport-associated anion current argued that the anion current recovery rate is much slower when EAAT2 operates in the exchange mode than the transport mode (Otis and Kavanaugh, 2000).

We determined the relative rates of the two transport modes using radiolabeled substrates in combination with a system based on solubilized glutamate transporters reconstituted in liposomes (Zhou *et al.*, 2013; Zhou *et al.*, 2014a). Our experiments and

computer simulations suggested that net glutamate uptake and glutamate exchange have similar rates (Zhou *et al.*, 2014a). Interestingly, we also found that the exchange process is voltage sensitive and that a sodium leak is present in EAAT2 due to transporter cycling from the outward to the inward conformation in the absence of glutamate (Zhou *et al.*, 2014a; for review of the molecular functioning of sodium-coupled symporters see: LeVine *et al.*, 2016).

Thus, the mismatch between D-aspartate accumulation and transporter distribution could not be explained simply by assuming that terminals perform rapid heteroexchange.

13. A larger loss of D-aspartate from astrocytes than from terminals?

Why did D-aspartate accumulate as fast in nerve terminals as in astroglia when hippocampal slices were incubated in D-aspartate (Furness *et al.*, 2008)? As outlined above, we do not know. One possibility that remains to be experimentally tested is if astrocytes lose D-aspartate faster than terminals (Fig. 5).

Although heteroexchange cannot explain the strong labeling of terminals (see Section 12 above), it might contribute to a weakening of the labeling of astrocytes by inducing release from astrocytes: The internal glutamate concentrations are far higher in terminals than in astrocytes (see Section 12 above). Consequently, D-aspartate molecules entering terminals will be more outnumbered by (diluted with) L-glutamate present in cytosol than D-aspartate molecules entering glia. As explained above (Section 12), substrates (L-glutamate, L-aspartate or D-aspartate) present in the extracellular fluid may induce exchange-mediated release of internal substrates. However, because of the higher [L-Glu]/[D-Asp] ratio, glutamate is more likely to bind to the transporter from the inside than D-aspartate. Consequently, heteroexchange can be expected to mostly result in release of glutamate from terminals. In contrast, internal D-aspartate in astrocytes will face less competition from glutamate and will therefore be expected to have a higher probability of being translocated out. Similarly, endogenous glutamate present on the outside (for instance glutamate that has been released from terminals) will mostly induce glutamate/glutamate exchange with terminals, and more frequently glutamate/D-aspartate exchange with glia. The net effect will be that D-aspartate taken up into terminals is likely to stay there, while some of the D-aspartate taken up into astrocytes is likely to be released resulting in an underestimation of the true uptake activity.

Additional factors that have been proposed to contribute to leaks from astrocytes are channels, such as unpaired connexons (acting as functional hemichannels), pannexins, P2X receptors, or volume regulated anion channels (Abudara *et al.*, 2015; Rovegno *et al.*, 2015; for review see: Malarkey and Parpura, 2008). This may be more significant in slices than in normal healthy tissue because tissue damage (e.g. cutting of slices) may activate connexins (Abudara *et al.*, 2015; Rovegno *et al.*, 2015). However, the combined conductance must be lower than the capacity of the astrocytes to maintain their transmembrane gradients considering that astrocytes in the hippocampal slice preparations are able to maintain their membrane potentials (Bergles and Jahr, 1997; Hanson *et al.*, 2015).

If D-aspartate taken up by astrocytes is partly lost due to a combination of mechanisms as suggested above, then this may obscure the real transport rate in astrocytes. D-aspartate is after all an artificial substrate.

14. EAAT2 is probably expressed in terminals belonging to a variety of neurons

EAAT2 mRNA is found in multiple neuronal populations (see Section 5.1 above for references). Further, D-aspartate uptake has been shown electron microscopically to occur in terminals, not only in the hippocampus as explained above, but also in the rat striatum (Gundersen *et al.*, 1996). Futher, there are several reports claiming that EAAT2 is expressed in axon-terminals in other parts of the CNS (Melone *et al.*, 2009; Melone *et al.*, 2011) in agreement with *in-situ* data (e.g. Berger and Hediger, 1998; Berger *et al.*, 2005).

Expression of EAAT2 in several types of axon-terminals is probable. There are many types of synapses with different properties. While most nerve terminals are too small to be studied directly with the patch-clamp technique, this is possible in some places. For instance, direct electrical recordings from retinal bipolar cell presynaptic terminals showed EAAT activation implying that they express functional transporters (Palmer *et al.*, 2003). In contrast, direct recordings from the Calyx of Held synapse in the medial nucleus of the trapezoid body (MNTB) in the brainstem (von Gersdorff and Borst, 2002) did not show any presynaptic EAAT activity (Billups *et al.*, 2013).

In agreement, when flox-EAAT2 mice are crossed with synapsin-Cre mice, then there is a reduction in glutamate uptake in synaptosomes in homogenates, not only from the hippocampus, but from the entire forebrain (Fig. 4A). This strong reduction cannot be explained by a loss of EAAT2 expression only in terminals from the CA3 pyramidal cells. To explain the loss of activity in a whole forebrain homogenate, there must be a more extensive loss of EAAT2. And as the total EAAT2 levels were virtually unchanged (as determined by Western blotting; Fig. 4B), then the most likely explanation is loss of EAAT2 from a large number of terminals in addition to those from CA3 pyramidal cells.

The realization that synaptosome preparations measures EAAT2 in nerve terminals more efficiently than EAAT2 in astrocytes, is important considering that synaptosomes have been widely used to determine brain glutamate uptake activity (Danbolt, 2001; Beart and O'Shea, 2007; Sheldon and Robinson, 2007). If the expression of EAAT2 in nerve terminals is regulated differently from EAAT2 in astrocytes (which is likely), then it may be worthwhile to reanalyze old reports based on synaptosomes (Hardy *et al.*, 1987; Procter *et al.*, 1988; Felipo *et al.*, 1989; Gilad *et al.*, 1990; Maddison *et al.*, 1996; Dixon and Hokin, 1998; Zhu *et al.*, 1998; Mitrovic *et al.*, 1999; Zhu *et al.*, 1999; Azbill *et al.*, 2000; Levenson *et al.*, 2000; Nogueira *et al.*, 2002; Xu *et al.*, 2003; Fontella *et al.*, 2004). Many of these findings have been forgotten after later studies have used methods that detect total tissue EAAT2 (e.g. immunoblots).

15. EAAT2 in terminals will short-circuit the glutamate-glutamine cycle

The physiological roles of EAAT2 in terminals remain to be determined. One obvious function would be direct recycling of transmitter glutamate (Fig. 5A).

The prevailing view, however, has been that glutamine released from astrocytes is the predominant source of glutamate in glutamatergic terminals (Van den Berg and

Garfinkel, 1971; Benjamin and Quastel, 1972; Hertz *et al.*, 1999; Sibson *et al.*, 2001) especially in situations of high demand such as persistent epileptiform activity (Tani *et al.*, 2010; Tani *et al.*, 2014). The idea has been that glutamate is taken up by astrocytes, converted to glutamine by the action of the astroglial enzyme glutamine synthetase (GS) in an ATP-dependent manner, released, taken up by axon-terminals and converted back to glutamate by means of phosphate activated glutaminase (Erecinska and Silver, 1990; Marcaggi and Coles, 2001; Hertz, 2013).

This hypothesis implies that the supply of glutamine to terminals keeps up with glutamate release and that may not always be the case (Waagepetersen *et al.*, 2005; Kam and Nicoll, 2007; Marx *et al.*, 2015). Further, a considerable fraction of glial glutamate is degraded via the TCA-cycle or by deamination (Bak *et al.*, 2006; McKenna, 2007; Pardo *et al.*, 2011; Marx *et al.*, 2015).

Glutamine can be released to the extracellular fluid, but the exact mechanisms are still being discussed. This may be by means of neutral amino acid transporters in the astrocytic membrane (Mackenzie and Erickson, 2004; Nissen-Meyer *et al.*, 2011; Hamdani *et al.*, 2012; Bhutia and Ganapathy, 2015), but there are several possible mechanisms including channels (for review see: Malarkey and Parpura, 2008).

There is also no consensus with respect to the mechanism whereby glutamine can enter the terminals. No glutamine transporter protein has so far been positively identified in terminals in brain tissue (Mackenzie and Erickson, 2004; Conti and Melone, 2006; Zhou and Danbolt, 2014; Grewal *et al.*, 2009). One possibility is that they have evaded detection in glutamatergic terminals due to methodological challenges. Another possibility is that they have not been detected simply because they are not there. A third possibility is that the focus has been on the wrong transporters. After all, there are at least 14 different solute carrier proteins with the ability to transport glutamine (Bhutia and Ganapathy, 2015). For instance SNAT7 (slc38a7) and SNAT8 (slc38a7) have recently been reported to be expressed in axon-terminals (Hagglund *et al.*, 2011; Hagglund *et al.*, 2015). In line with this, there is electrophysiological evidence for glutamine uptake at the Calyx of Held synapse (Billups *et al.*, 2013). As mentioned above (see Section 14), this synapse is special with a giant nerve terminal and may therefore not be representative for the majority of synapses. A problem with the majority of synapses is that the boutons are so small that they are difficult to study (von Gersdorff and Borst, 2002), and as a consequence, we know less about them.

Other sources of transmitter glutamate comprise *de novo* synthesis (Hassel and Bråthe, 2000; McKenna *et al.*, 2000), and, as described in this review, direct uptake by EAAT2 glutamate transporters in the terminals themselves (Furness *et al.*, 2008). The relative contributions of the various mechanisms are still debated and a complicating factor is that this may differ between brain regions and in disease. Marx and co-workers has recently tried to put available quantitative data together (Marx *et al.*, 2015). This is an informative an easy to read review and an excellent guide for further data collection and discussions.

16. Conclusions

The total content of EAAT2 in the normal young adult rat brain is about 1 mg/gram tissue and about 100 times higher than that of EAAT3. The levels of EAAT4 are even lower than those of EAAT3. EAAT2 accounts for around 95 % of the total glutamate

uptake activity, and complete lack of EAAT2 results in a serious phenotype starting (in the mouse) at the end of the second week with lower body weight and hyperactivity developing into spontaneous epilepsy and increased lethality from the third week. About 90 % of EAAT2 is astroglial and about 10 % is present in axon-terminals in the rat hippocampus CA1. This explains the high EAAT2 mRNA levels in CA3 pyramidal neurons. Selective deletion of EAAT2 in astrocytes gives a phenotype resembling that of the global knockout, while selective deletion in neurons has as yet unidentified consequences implying that most of the brain glutamate uptake is due to EAAT2 in astrocytes. EAAT3 is selectively expressed in neurons, but expressed at much lower levels that EAAT2 and targeted to the somato-dendritic compartments while neuronal EAAT2 is targeted to the axon-terminals. More than half of the uptake measured in synaptosome preparations is due to EAAT2 in terminals while most of the remainder is due to EAAT2 in glia. EAAT2 in terminals would be expected to make a contribution to the recycling of glutamate (and thereby short-circuit the so called glutamate-glutamine cycle), but how important this is compared to other sources of glutamate remains to be determined. Most of these data, however, are from the hippocampus so more work is required before we can be absolutely sure about the presence of EAAT2 in terminals in other brain regions. EAAT4 is also selectively neuronal, but only expressed in cerebellar Purkinje cells and in scattered neurons in the cerebral cortex, while EAAT1 is selectively astrocytic in the brain. After all these years, we do not yet have a complete picture of what physiological roles the various glutamate transporters play.

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Figure legends

Figure 1. Diagrammatic representation of lesions of the neocortex made to destroy the cell bodies that give rise to the glutamatergic corticostriatal fibers. When the neocortex is removed, the axon-terminals projecting to the striatum degenerate. This results in a strong reduction in synaptosomal glutamate uptake activity in striatum on the lesioned side. However, there is also a reduction in the expression of EAAT1 and EAAT2 in the striatum (Levy *et al.*, 1995). This motivated investigation into whether neurons influence expression of EAAT2 in astrocytes (see Fig 2). (Modified from a figure provided by Line ML Boulland. Copyright: Neurotransporter AS, Oslo, Norway; Reproduced with permission).

Figure 2. When cultured separately (upper row), glial cells do not express EAAT2, while neurons do. On the other hand, if neurons are cultured together with astrocytes, then EAAT2 expression is turned on in astrocytes and decreased in neurons. This influence does not require direct cell-to-cell contact, as the stimulating effect is seen after transferring cell free conditioned medium from neuronal cultures to the glial ones (Gegelashvili *et al.*, 1996; Gegelashvili *et al.*, 2000; Plachez *et al.*, 2000; Martinez-Lozada *et al.*, 2016). (Copyright: Neurotransporter AS, Oslo, Norway; Reproduced with permission).

Figure 3. Evidence for neuronal expression of EAAT2 in terminals and synaptosomes. Panel a: A section from a hippocampal slice incubated in D-Asp, fixed and embedded for transmission electron microscopy and immunogold labelled for D-Asp uptake. Half of the gold particles are in a nerve terminal (t) which is forming a synapse with a spine (sp). Other particles lie over astrocyte profiles. Scale bar = 200 nm. Panel b: Immunogold labelling for EAAT2 localizes to the membranes of an isolated synaptosome (arrows) identifiable by the presence of vesicles internally (syn). Other membrane profiles that can be seen to be labelled are probably derived from glia. Scale bar = 200 nm. Panel c: After incubation of synaptosome preparations with D-Asp, immunogold labelling of the latter reveals uptake into synaptosomes, identifiable as before by vesicles internally (syn) and by the presence of a spine fragment forming an intact synapse with the synaptosome. Scale bar = 100 nm. From the same material as in (Furness *et al.*, 2008) (Copyright: Neurotransporter AS, Oslo, Norway; Reproduced with permission).

Figure 4. Selective deletion of EAAT2 in neurons resulted in a major reduction (to about half) of the glutamate uptake activity in a crude synaptosome containing homogenate prepared from the forebrain (A). GABA uptake was normal in all cases. Despite the reduction in uptake activity in the forebrain homogenate, Western blots (**B**) showed that the total EAAT2 levels were virtually unchanged. Note that the synapsin-Cre mice do not express Cre in all of their neurons (Y. Zhou and N.C. Danbolt, manuscript in preparation). Consequently, the observed reduction represents an underestimation. Method: mice with floxed EAAT2-gene (Zhou *et al.*, 2014b) were crossed with synapsin-Cre mice (Zhu *et al.*, 2001; Jackson Laboratories, cat.no 003966). The Western blot with the above homogenates (**B**) was probed with anti-B493 antibodies to EAAT2 (RED; antibody-id #8; Sheep; Li *et al.*, 2012) and with anti-A522 antibodies to EAAT1 (GREEN;

antibody-id #314; Rabbit; Holmseth *et al.*, 2009). (Copyright: Neurotransporter AS, Oslo, Norway; Reproduced with permission).

Figure 5. Schematic illustration of (**A**) *in vivo* handling of glutamate (Glu) and (**B**) uptake of D-aspartate (D-Asp) when hippocampal slices is incubated with D-Asp. **Panel A:** Glutamate present in the extracellular space may be taken up into terminals by means of EAAT2 directly or via EAAT2 (and EAAT1) present in astrocytes and subsequent relocation. Astrocytes have higher densities of EAAT2 than terminals (Table 1) and the more serious consequences of deleting EAAT2 in astrocytes than in neurons suggest that astrocytes *in vivo* take up more glutamate than terminals (Petr *et al.*, 2015). Glutamate taken up into astrocytes may be converted to glutamine by the action of the enzyme glutamine synthetase (GS) or be degraded (e.g. via the TCA-cycle; McKenna, 2007). The glutamine produced can be released to the extracellular fluid, taken up neurons and converted back to glutamate. However, there is no consensus with respect to the glutamine transporters involved. Other sources of transmitter glutamate comprise *de novo* synthesis (Hassel and Bråthe, 2000; McKenna *et al.*, 2000), and, as described in this review, direct uptake by EAAT2 glutamate transporters in the terminals themselves (Furness *et al.*, 2008). The relative contributions of the various mechanisms are still debated and a complicating factor is that this may differ between brain regions and in disease. The contribution by EAAT3 is believed to be minor due to lower expression levels (Holmseth *et al.*, 2012b). **PANEL B:** When hippocampal slices are incubated with D-Asp, then D-Asp is taken up by a combination of net uptake and heteroexchange. D-Asp will start accumulating in both terminals and glia with apparently similar rates despite much higher transporter densities in the latter. However, in contrast to glutamate, D-Asp will remain D-Asp because it is hardly metabolized. Once inside terminals, D-Asp will be diluted in excess glutamate. Therefore, when an internal substrate is exchanged with external D-Asp, then it will mostly be internal glutamate rather than D-Asp that goes out. In contrast, internal D-Asp in glia will have a higher probability of going out simply because there is less of the alternative (namely glutamate which has been metabolized). Further, any glutamate released from terminals will for the same reason tend to deplete glia more than terminals. If there should be some disconnection of gap junctions (to form hemichannels) or activation of volume activated anion channels, then this will also contribute to making the loss of D-Asp from glia higher than the loss from terminals. (Copyright: Neurotransporter AS, Oslo, Norway; Reproduced with permission).

Figure1

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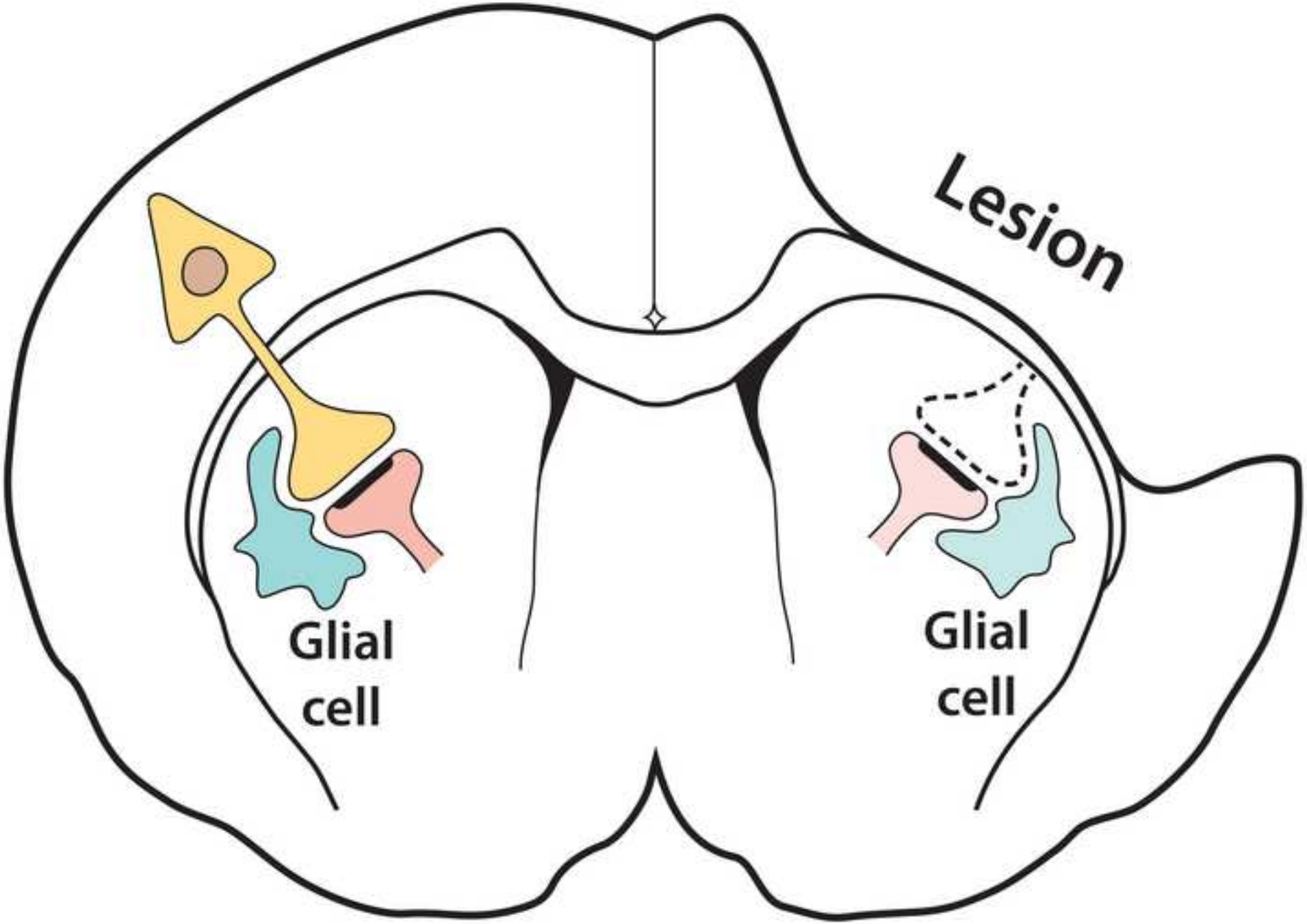

Figure2

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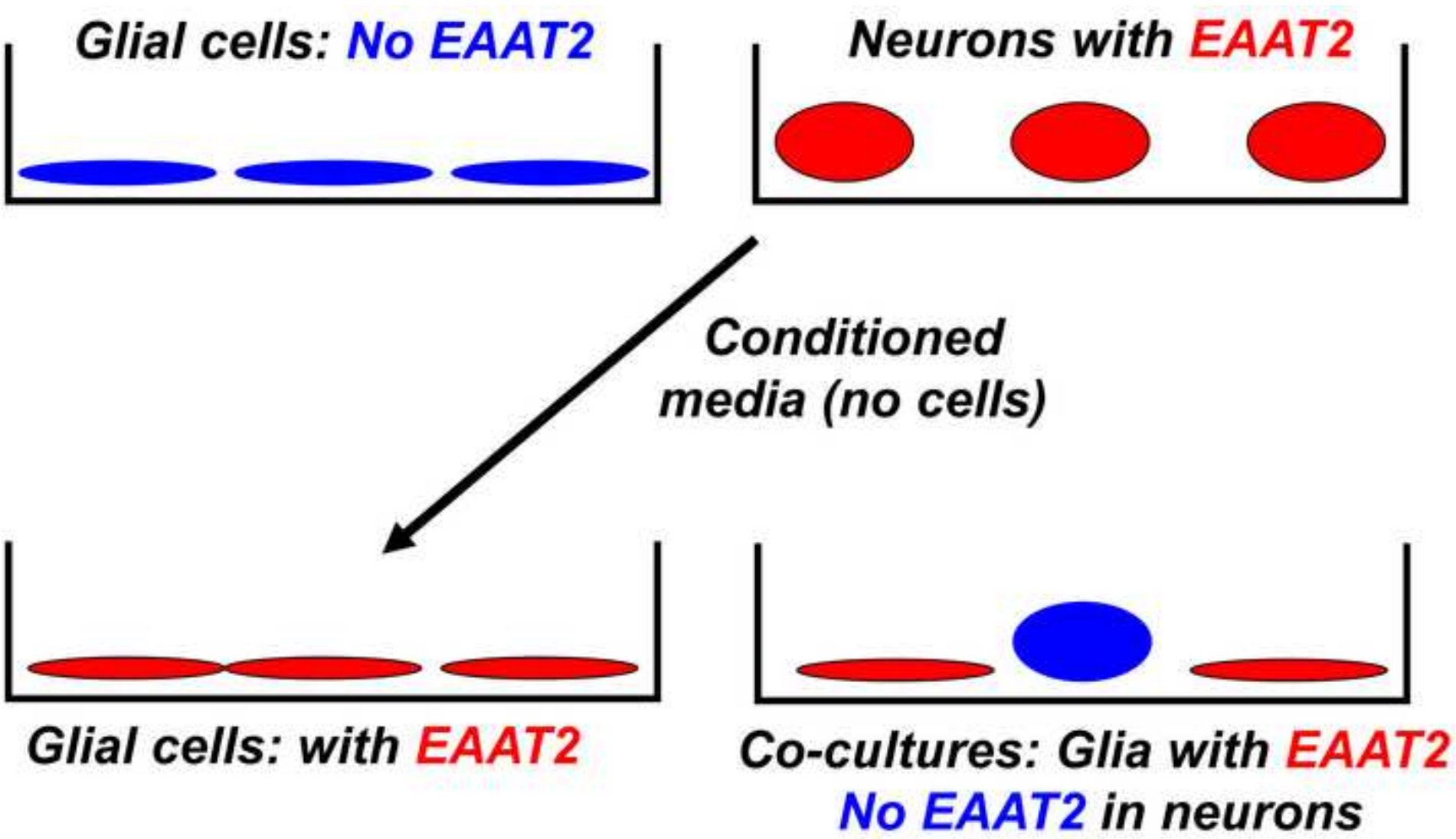

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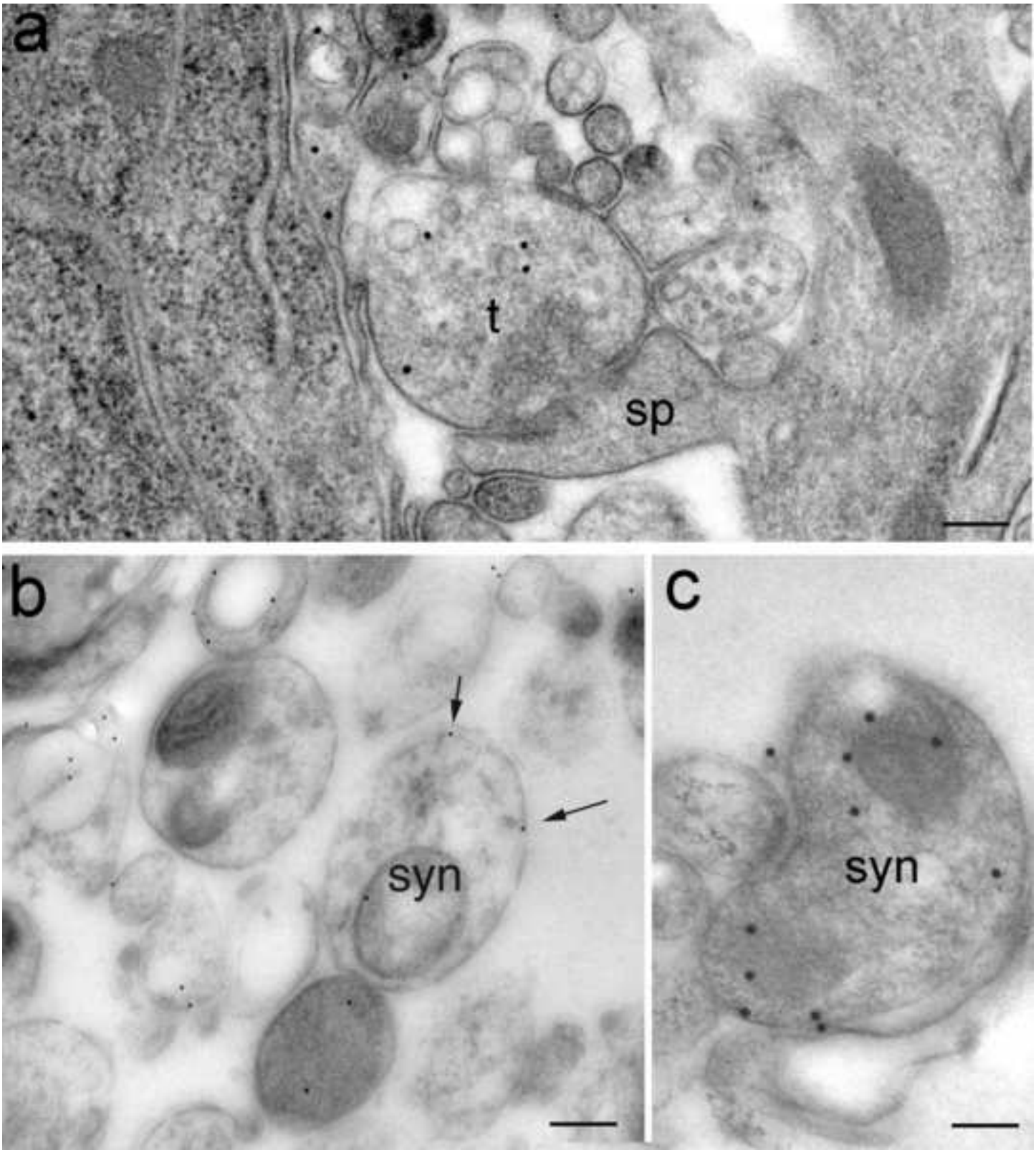

Figure4

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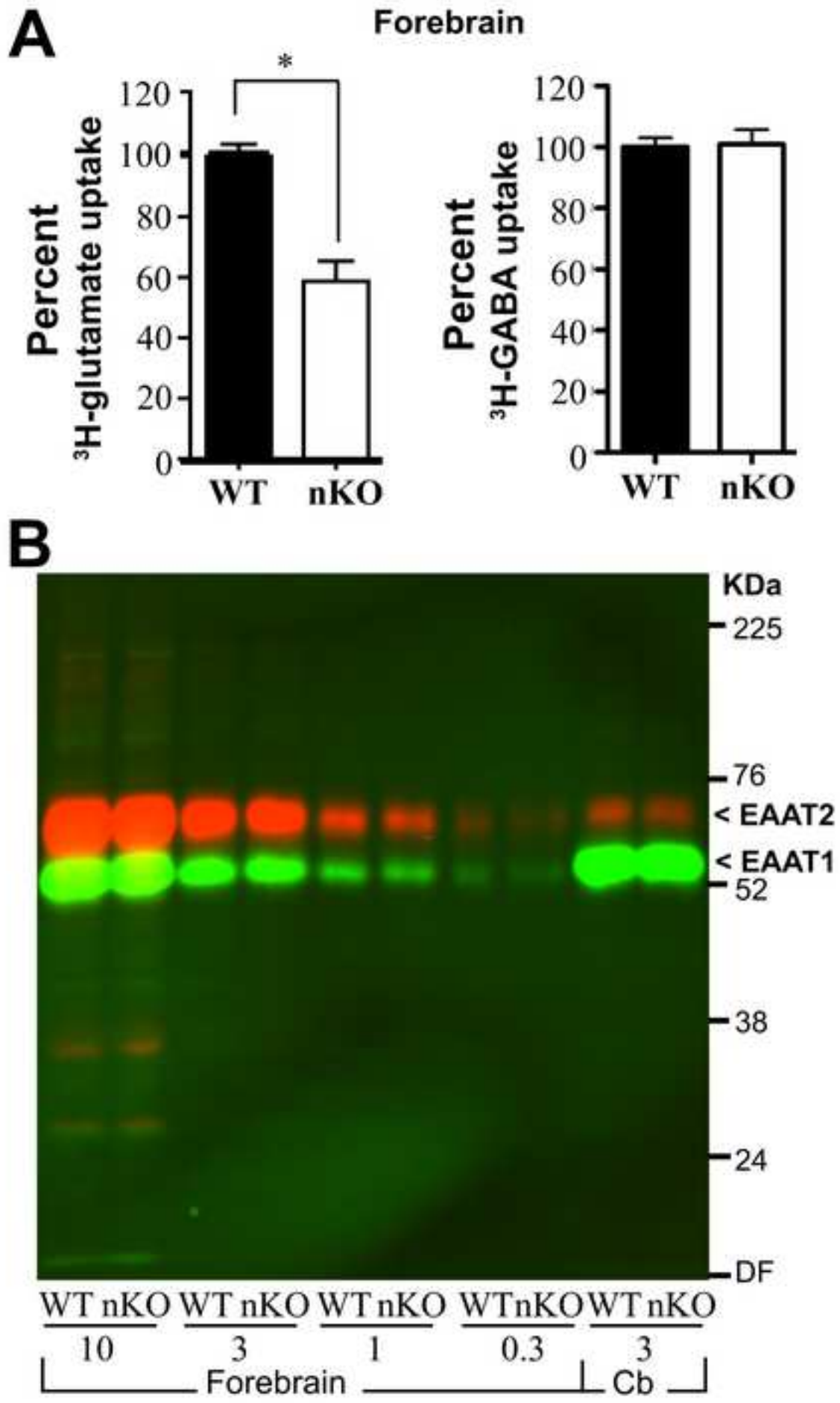

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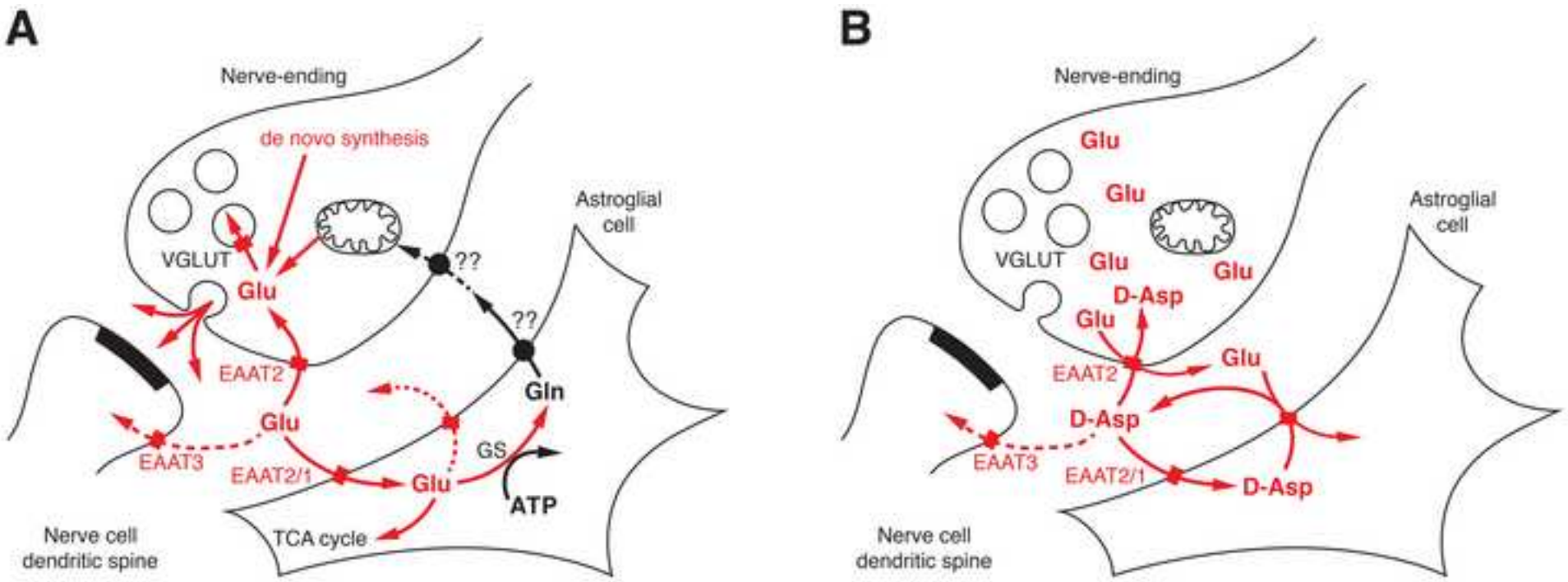

Table 1. Tissue concentrations of glutamate transporters in the hippocampus (stratum radiatum, CA1) of young adult Wistar rats

	mg EAAT protein per g tissue	µmol EAAT protein per liter	EAAT molecules per μm^3 tissue	Plasma membranes per tissue volume ($\mu m^2/\mu m^3$)	Location	Maximum EAAT molecules per μm^2 plasma membrane
EAAT1	0.32	5.3	3200	1.4	Astroglia	2300
EAAT2	1.3	21	12000	1.4	Astroglia	7500
				~1	Terminals	750
EAAT3	0.013	0.22	130	1.5	Dendrites	90

The above data only give an indication of the average transporter densities because expression levels are subject to regulation (Martinez-Lozada *et al.*, 2016). For instance, EAAT3 is to a large extent present intracellularly in the brain while most of EAAT2 and EAAT1 are at the surface (for details see the original articles: Dehnes *et al.*, 1998; Lehre and Danbolt, 1998; Furness *et al.*, 2008; Holmseth *et al.*, 2012b).