Glutamate and GABA Transporters in Mesial Temporal Lobe Epilepsy

By

Lars Petter B-W Bjørnsen

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Department of Anatomy
Institute of Basic Medical Sciences
University of Oslo
Oslo, Norway

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Abbreviations

ALT = Alanine transaminase
CA = Ammon's horn
EAAC = EAAT3 = EAAC1
EAAT = Excitatory amino acid transporter
GABA = gamma amino butyric acid
GAD = Glutamate dehydrogenase
GAT = GABA transporter
GLAST = Glutamate-aspartate-transporter = EAAT1 (slc1a3)
Gln = Glutamine
GLT-1 = Glutamate transporter = EAAT2 (slc1a2)
Glu = Glutamate
GLUT1 = Glucose transporter 1 (slc2a1)
GS = Glutamine synthetase
LDH = Lactate dehydrogenase
ML = Molecular layer
MTLE = Mesial temporal lobe epilepsy
NMDA = Ionotropic glutamate receptor (N-methyl-D-aspartate)
PAG = Phosphate-activated glutaminase
PC = Pyruvate carboxylase
PDH = Pyruvate dehydrogenase
PTLE = Paradoxical temporal lobe epilepsy
SDS = Sodium dodecyl sulfate
SDS-PAGE = Sodium dodecyl sulfate – polyacrylamide gel electrophoresis
SNAT = Sodium-neutral amino transporter (glutamine transporter)
TLE = Temporal lobe epilepsy

List of papers

Paper 1
Lee TS, Bjørnsen LP, Paz C, Kim JH, Spencer SS, Spencer DD, et al. GAT1 and GAT3 expression are differently localized in the human epileptogenic hippocampus. Acta Neuropathol. 2006;111(4):351-63.

Paper 2
Bjørnsen LP, Eid T, Holmseth S, Danbolt NC, Spencer DD, de Lanerolle NC. Changes in glial glutamate transporters in human epileptogenic hippocampus: inadequate explanation for high extracellular glutamate during seizures. Neurobiol Dis. 2007;25(2):319-30.

Paper 3
Bjørnsen LP, Ghezu M, Zhou Y, Danbolt NC, Sonnewald U. The GLT-1 (EAAT2; slc1a2) glutamate transporter is essential for glutamate homeostasis in the neocortex of the mouse. J. Neurochem 2013;128(5):641-649

Paper 4
Holmseth S, Dehnes Y, Bjørnsen LP, Boulland JL, Furness DN, Bergles D, et al. Specificity of antibodies: unexpected cross-reactivity of antibodies directed against the excitatory amino acid transporter 3 (EAAT3). Neuroscience. 2005;136(3):649-60.

Additional paper not included in this thesis:

Eid T, Lee TS, Thomas MJ, Amiry-Moghaddam M, Bjørnsen LP, Spencer DD, et al. Loss of perivascular aquaporin 4 may underlie deficient water and K+ homeostasis in the human epileptogenic hippocampus. Proc Natl Acad Sci U S A. 2005;102(4):1193-8.

Summary

GABA and glutamate transporter proteins are important in relation to epilepsy because they represent the mechanisms that keep the extracellular concentrations of GABA and dicarboxylic amino acids low. GABA and glutamate are the major inhibitory and excitatory neurotransmitters, respectively, in the brain. Consequently, these transporters are major players in enabling the brain to balance inhibition and excitation. In fact, by microdialysis it has been reported changes in extracellular glutamate and GABA levels prior to and during seizures, and it has been postulated that changes in the expression or function of glutamate and GABA transporters may be the cause of the extracellular glutamate excess. Papers 1 and 2 investigate the expression and distribution of GABA transporters (GAT1 and GAT3) and glutamate transporters (GLAST and GLT-1) in the hippocampus from humans with temporal lobe epilepsy (TLE). Epilepsy comprises a heterogeneous group of disorders reflecting underlying brain dysfunction. Temporal lobe epilepsy (TLE) is one of the most common chronic seizure disorders and is the most intensely studied subtype. Temporal lobe epilepsy is divided into mesial temporal lobe epilepsy with hippocampal sclerosis (MTLE), mass-associated temporal lobe epilepsy (MaTLE), and paradoxical temporal lobe epilepsy (PTLE). Hippocampal sclerosis is believed to play a key role in the increased extracellular glutamate concentration and generation of seizures in MTLE. However, it is not known precisely which cell populations, when lost due to sclerosis, cause the network imbalance. As shown in the present thesis, there was no significant overall change in the expression of the main GABA transporters GAT1 and GAT3, but immunohistochemical staining showed regional differences in GAT1 immunoreactivity within the hippocampus. Similarly, the main glutamate transporters, GLAST and GLT-1, showed no overall significant difference in the expression between the non-MTLE and MTLE hippocampi in Paper 2. However, there was a difference in the pattern of immunolabeling for GLAST and GLT-1. This study does not support the role of glutamate transporters as a cause of elevated levels of extracellular glutamate in the epileptogenic hippocampi, but undetected changes in the splice variants of the transporters or unexpected antibody cross-activity may lead to biased or even false results (Paper 4). In Paper 3 we investigated the glutamate homeostasis in mice lacking the GLT-1 protein. ^{13}C magnetic resonance spectroscopy (MRS) of cortical tissue from GLT-1 knockout mice was performed following intraperitoneal injection of ^{13}C-labeled glucose and acetate. Metabolite levels were analyzed from the neocortex and cerebellum. Compared with wild-type mice, GLT-1 knockout mice had normal levels of glutamate and glutamine in the cerebellum, but they exhibited decreased levels in the neocortex. The findings suggest that GLT-1 in cortical nerve terminals may contribute significantly to the replenishment of the pool of transmitter glutamate thereby short-circuiting the glutamate-glutamine cycle. In conclusion, we did not observe any significant overall changes in the expression of glutamate or GABA transporters in human sclerotic hippocampi, but did observe regional changes. Further, the findings suggesting that GLT-1 in nerve terminals is a major player that needs to be followed up when conditional knockout mice become available. The role of glutamate and GABA transporters in temporal lobe epilepsy remains elusive.

Introduction

Approximately 50 million individuals have epilepsy worldwide. The prevalence ranges between 4 to 8/1,000 (Hauser et al., 1991, Svendsen et al., 2007). This statistic makes it one of the most common neurological disorders. During a lifespan, one of ten individuals will experience an epileptic seizure, and one third of these individuals will develop epilepsy (Engel and Pedley, 2008). A seizure is commonly referred to as a hyperexcitability and hypersynchrony of neuronal networks, which are intrinsically hyperexcitable and fire synchronously and excessively (Margineanu, 2010). The definition does not include any pathogenetic explanation because the question of what causes epilepsy and epileptic seizures is, to a large degree, still unanswered. Epilepsy is a heterogeneous group of disorders reflecting underlying brain dysfunction. It may be caused by genetic abnormalities, birth damage, prolonged febrile seizures, infections, stroke, or tumors. Epilepsy is defined as a chronic condition of the brain characterized by an enduring propensity to generate epileptic seizures and by the neurobiological, cognitive, psychological, and social consequences of this condition (Fisher et al., 2005). An epileptic seizure is a transient occurrence of signs and/or symptoms due to abnormal and excessive neuronal activity in the brain (Fisher et al., 2005).

Temporal lobe epilepsy

One type of focal epilepsy is temporal lobe epilepsy (TLE), in which seizures originate from the temporal lobe. Although there are many types of genetic and acquired forms of epilepsy, TLE is among the most common of the chronic seizure disorders (Hauser and Kurland, 1975, Engel, 2001) and is the seizure disorder that is most intensely studied. TLE was defined in 1985 by the International League Against Epilepsy (ILAE) as a condition characterized by recurrent unprovoked seizures originating from the medial or lateral temporal lobe. Despite a wealth of descriptive data obtained from patient histories, imaging studies, electroencephalographic recordings, depth recordings before and during surgery, and histological studies of surgical and autopsy tissues, the epileptogenic process remains poorly understood. It is believed that when the hippocampus is asymmetrically atrophic on initial MRI, the shrunken hippocampus is a likely source of epileptic seizures in TLE (Engel, 2001). The presence of a shrunken hippocampus is also a predictive indicator of the medically refractory state of the disease (Semah et al., 1998). Depth electrode recordings demonstrate hypersynchronous electrical activity in the hippocampus that is often associated with auras that can spread and cause clinical seizures (Engel, 2001). Because this form of epilepsy is often intractable (Chadwick, 1990), many of these patients undergo an anteromedial temporal lobectomy with hippocampectomy for seizure control (Spencer et al., 1984). The condition seems to be medically intractable in approximately 40 % of cases, and surgical resection of the epileptic focus is the most efficient way to control the seizures.

Classification of temporal lobe epilepsy

TLE is divided into three broad categories, namely, mesial temporal lobe epilepsy (MTLE), mass-associated temporal lobe epilepsy (MaTLE), and paradoxical temporal lobe epilepsy (PTLE) (de Lanerolle et al., 2003). Approximately 70 % of patients with TLE who undergo surgical resection are classified as having MTLE. The hallmark of

MTLE is hippocampal sclerosis, which is characterized by neuronal loss and gliosis in the CA1, CA3, and hilar regions and reorganization of synaptic connections (Sommer, 1880, de Lanerolle and Lee, 2005). The sclerotic hippocampal formation is the most probable origin of seizures, and more than 85 % of patients become seizure-free after removal of the these regions (de Lanerolle et al., 2003). The clinical records of MTLE patients typically include an insult, such as febrile seizures, a brain trauma, or a brain infection that predates seizure onset by several years (Mathern et al., 1996). The remaining 30 % of patients with TLE who undergo surgery do not exhibit hippocampal sclerosis. Approximately half of these patients have a mass lesion outside the hippocampus, but within the temporal lobe, and are described as having mass-associated temporal lobe epilepsy (MaTLE) (de Lanerolle and Lee, 2005). The seizures seem to be generated by the mass, and the surgical outcome is dependent on the type of lesion. The other 50 % of patients have paradoxical temporal lobe epilepsy (PTLE) with an unknown etiology. For PTLE patients, the prognosis after surgery is not as good as that for patients with MaTLE and MTLE (de Lanerolle et al., 2003). MaTLE and PTLE are referred to as non-MTLE.

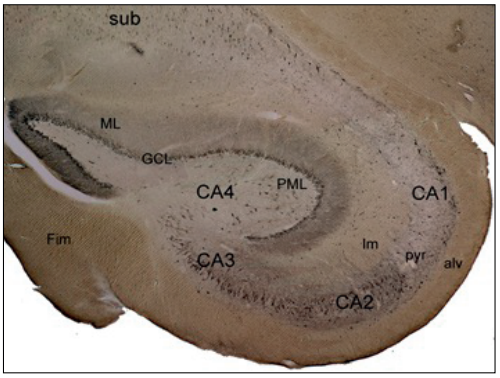

Figure 1
Photomicrographs of glutamate transporter EAAT3 (EAAC) immunoreactivity in the MTLE hippocampus. This low power view of the entire cross-section of a MTLE hippocampus shows the hallmarks of hippocampal sclerosis. The immunoreactivity is seen in the granule cell layer (GCL), in the molecular layer (ML), throughout the Ammon's horn (CA4, CA3, CA2, CA1) in the pyramidal cells, and in the subiculum. The hippocampus is characterized by pronounced neuronal loss in the CA1, CA3, and dentate hilus (CA4) regions; thus, there is less labeling in these regions. The CA2 region and subiculum are spared. Only neurons are labeled (granule and pyramidal cells). Pyr, stratum pyramidale; lm, lacunosum moleculare; alv, alveus; fim, fimbra. (L.P. Bjørnsen and T. Eid, unpublished)

Hippocampal sclerosis is thought to play a key role in the generation of seizures in MTLE (Mathern et al., 1997, de Lanerolle and Lee). However, the exact cell types that cause the network imbalance and seizure discharges are not known. There is also a lack of an effective drug treatment that both points to the identity of the defective component and corrects the network imbalance to an extent that produces symptomatic improvement (Sloviter, 2005). If the assumption that the hippocampus is a frequent source of seizures is correct, the observation that typical hippocampal sclerosis involves an extensive loss of dentate hilar neurons and the CA1 and CA3 pyramidal cells (Meldrum, 1994) logically focuses attention on surviving dentate granule cells (Tauck and Nadler, 1985, Nadler, 2003) and subicular neurons (Cohen et al., 2002) as likely seizure generator candidates. One hypothesis ("epileptogenic" mossy fiber sprouting) posits that a trauma- or seizure-induced loss of vulnerable dentate hilar neurons causes granule cells to redirect their axonal output to each other, resulting in a recurrent excitatory network (Tauck and

Nadler, 1985, Nadler, 2003). A second hypothesis ("inhibitory" mossy fiber sprouting) focuses on the post-synaptic targets of vulnerable hilar neurons, which include both granule cells and inhibitory neurons (Sloviter, 1992).

The role of glutamate and GABA in mesial temporal lobe epilepsy

Current evidence points to a complex role of glutamate and GABA in MTLE. Glutamate is the major mediator of excitatory signals in the mammalian central nervous system (Fonnum, 1984, Danbolt, 2001), while γ-aminobutyric acid (GABA) is the most abundant inhibitory neurotransmitter (Zachmann et al., 1966). Glutamate is almost exclusively located inside cells, primarily in nerve terminals (Storm-Mathisen et al., 1992, Ottersen et al., 1996), and the concentration inside synaptic vesicles may be as high as 100 mmol. The intracellular localization is essential because glutamate is relatively inactive as long as it is intracellular and not able to bind to receptors on the outside of the cellular membrane. The amounts of glutamate in brain tissue is high (5-15 mmol per kg brain tissue) (Schousboe, 1981), and more than 1,000-fold higher than the concentration required to activate glutamate receptors. The normal concentration of glutamate in the extracellular fluid is low, possibly as low as 25 nM (Kugler and Schmitt, 1999, Herman and Jahr, 2007, Zhou and Danbolt, 2013). Glutamate homeostasis depends on the appropriate control of glutamate metabolism (synthesis and degradation) and transport (release and uptake), and it is a prerequisite for normal brain function. A prolonged increase in extracellular glutamate triggers the death of neurons. The mechanisms that are in place to maintain glutamate homeostasis are quite robust and normally serve to prevent a detrimental increase in glutamate concentration. The handling of glutamate in the brain depends on a sophisticated interplay between neurons and astrocytes (for a review, see (Danbolt, 2001, Zhou and Danbolt, 2013)).

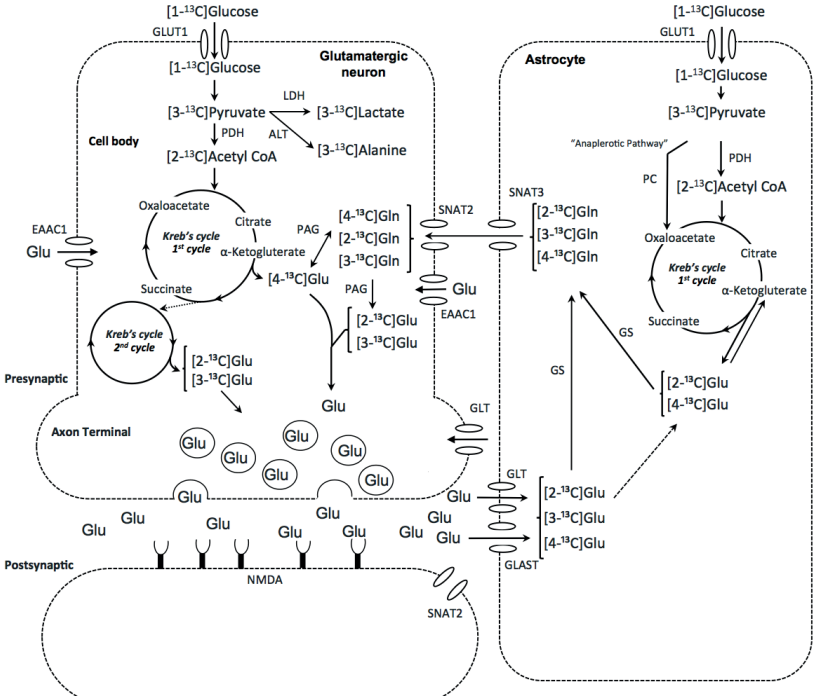

Figure 2

Schematic presentation of a glial neuronal metabolic interaction following the uptake of [1-^{13}C]glucose (Sonnewald and McKenna, 2002). [1-^{13}C]glucose enters glycolysis in both neurons and astrocytes to form one molecule of [3-^{13}C]pyruvate, which can then be converted to [3-^{13}C]lactate or [3-^{13}C]alanine in the cytosol. Alternatively, it can enter the mitochondria and be converted to [2-^{13}C]acetylCoA, or it can be converted to oxaloacetate via pyruvate carboxylase (PC), an astrocyte-specific enzyme (Shank et al., 1985). One-third of the acetylCoA from glucose is formed in the neuronal mitochondria (Hassel and Sonnewald, 1995a, Qu et al., 2000a), thus, it is assumed that [1-^{13}C]glucose metabolism is primarily neuronal. [2-^{13}C]acetylCoA condenses with oxaloacetate to form TCA (Kreb's) cycle intermediates, including [2-^{13}C]citrate and then [4-^{13}C]α-ketoglutarate. The ^{13}C label can exit the TCA cycle from [4-^{13}C]α-ketoglutarate as [4-^{13}C]glutamate or, alternatively, continue in the cycle. Upon release, [4-^{13}C]glutamate is taken up either back into the terminals (Danbolt, 2001, Waagepetersen et al., 2005, Olstad et al., 2007) or into astrocytes (Schousboe and Hertz, 1981, Danbolt, 2001, Huang et al., 2004), where it can be converted to [4-^{13}C]glutamine by the enzyme glutamine synthetase (GS) or to [3 or 2-^{13}C]glutamine after passage through the TCA cycle. Glutamine is then released into the extracellular space by a sodium-neutral amino transporter in the astrocytic membrane, SNAT3 (Boulland et al., 2002, Boulland et al., 2003, Mackenzie and Erickson, 2004, Nissen-Meyer et al., 2011). Glutamatergic neurons can take up glutamine via the SNAT2 (SAT2; slc38a2) located in dendrites and cell bodies (Jenstad et al., 2009). It is currently not clear whether terminals have a functionally significant ability to take up glutamine (Conti and Melone, 2006). Neuronal mitochondria can convert glutamine back to glutamate by phosphate-activated glutaminase (PAG). Abbreviations: ALT: alanine transaminase; GAD: glutamate dehydrogenase; GLT-1: Glutamate Transporter 1 (Excitatory Amino Acid Transporter 2 – EAAT2 or slc1a2); GLAST: Glutamate Aspartate Transporter (Excitatory Amino Acid Transporter 1 – EAAT1 or slc1a3); Glu: glutamate; Gln: glutamine; GS: glutamine synthetase; LDH: lactate dehydrogenase; NMDA: ionotropic glutamate receptor (N-methyl-D-aspartate); GLUT1: Glucose transporter 1 (slc2a1); PAG: phosphate-activated glutaminase; PC: pyruvate carboxylase; PDH: pyruvate dehydrogenase. (Figure is based on Bjørnsen et al., 2014)

GABA is a multifaceted compound, and in addition to its role as a neurotransmitter, it serves as an intermediate in energy metabolism and amino acid homeostasis. Neuronal excitability is controlled by two different inhibitory mechanisms mediated through synaptic and extrasynaptic GABA receptors (Rossi and Hamann, 1998, Farrant and Nusser, 2005). The mechanisms causing dysfunctional neurotransmitter homeostasis are not clear; perhaps the most pertinent question is whether the changes in neurotransmitters are the causes or the consequences of long-standing epilepsy. It is well known that glutamate is associated with seizures, as substantiated by the convulsive effects of glutamate agonists, the anticonvulsive effects of glutamate antagonists, and the increased release of glutamate during seizures (Bradford, 1995). The glutamate analogue kainate is widely used to induce epileptic seizures and epilepsy in animal experiments (Ben-Ari, 1985), while another kainate derivative, the toxin domoic acid, can cause status epilepticus and epilepsy in humans (Teitelbaum et al., 1990, Cendes et al., 1995). Furthermore, several new antiepileptic drugs have been shown to act by reducing glutamate-mediated excitatory neurotransmission via the NMDA (felbamate) and AMPA/kainite (topiramate) receptors (White et al., 2007). Many drugs suppress the release of glutamate by enhancing the inhibitory effects of GABA (Perucca, 2005). The results from measurements of glutamate content in the hippocampus have been conflicting. Low glutamate content has been observed in both humans and animal models of TLE (Koyama, 1972, Peeling and Sutherland, 1993, Petroff et al., 2002b), while a study employing in vivo microdialysis and depth electrode EEG reported elevated extracellular glutamate interictally in the epileptogenic vs. non-epileptogenic human hippocampus (Cavus et al., 2008).

In 1993, During and Spencer demonstrated an increased extracellular level of glutamate in the hippocampal formation in patients with MTLE prior to and during seizures using microdialysis (During and Spencer, 1993). The increased extracellular level of glutamate remained elevated for several minutes after the cessation of seizure activity, indicating a reduced capacity for glutamate clearance. In a study of MTLE hippocampi, the extracellular level of glutamate was also found to be interictally elevated, i.e., in the period between seizures, supporting a continuous impairment of glutamate homeostasis (Cavus et al., 2005). Increased release and extracellular levels of glutamate lead to excessive stimulation of glutamate receptors, which may cause neuronal death and may be a final common pathway leading to several neurological diseases (Lipton and Rosenberg, 1994). By inducing cell death and associated structural changes, such as the formation of novel synapses and aberrant connections between cells, glutamate may contribute to the development of the hyperexcitable network associated with MTLE. Intracellular recordings from slice preparations of surgical specimens show that the dentate granule cells are hyperexcitable in MTLE. The hyperexcitability of granule cells is glutamate-dependent (Williamson and Spencer, 1994) and possibly related to the sprouting of recurrent collaterals that is known to occur (Babb et al., 1991). The latter finding is in line with the idea that perturbed glutamate homeostasis could be essential not only for the inductions of individual seizures, but also for the generation of the underlying structural and functional changes in the epileptic focus.

As mentioned earlier, a major proportion of glutamate in the brain is contained within glutamatergic neurons. In MTLE, there is an extensive loss of glutamatergic pyramidal neurons in the hippocampal formation. However, according to studies of surgical specimens, there is no correlation between glutamate content and neuronal loss in the hippocampal formation of patients with MTLE (Petroff et al., 2002a). Some hippocampi with the greatest neuronal loss contain a larger amount of glutamate than hippocampi with moderate neuronal loss. The lack of correlation between glutamate content and cell loss could reflect increased glutamate content in the surviving glutamatergic neurons and/or in astrocytes and GABAergic neurons.

A theory of an imbalance in the glutamate-to-GABA ratio causing epileptic seizures is supported by the fact that several drugs that suppress glutamatergic or enhance GABAergic action are efficient antiepileptic drugs (Petroff et al., 2002a). However, such drugs can also have paradoxical effects, aggravating seizures in patients with certain epilepsy types, suggesting that the explanation of unbalanced excitation and enhanced inhibition may underlie epileptic seizures (Wilson and Engel, 1993, Nusser et al., 1998). Specifically, it has been proposed that increased GABAergic inhibition may underlie epileptiform hypersynchrony (Isokawa-Akesson et al., 1989). This hypothesis is supported by findings of GABA-dependent spontaneous rhythmic activity in brain slices of the human epileptic hippocampus (Schwartzkroin and Haglund, 1986). In addition, GABA may be excitatory in some settings, as has been demonstrated in the subiculum (a region of hippocampal output) of MTLE patients, where depolarizing GABA responses appeared to contribute to interictal epileptiform activity (Cohen et al., 2002). Extracellular GABA rises during and after seizures in the epileptogenic hippocampi, with interictally unchanged levels compared with those in non-MTLE hippocampi (During and Spencer, 1993, Treiman, 2001, Pan et al., 2008). In contrast, it has been shown that injecting mice with 3-nitropropionic acid (3-NPA) causes seizures by selectively inhibiting the TCA cycle in GABAergic neurons (Hassel and Sonnewald, 1995b, Zuchora et al., 2001). Furthermore, measurements of GABA in tissues have been inconclusive. Increased, unaltered, and decreased amounts have been reported in MTLE biopsy material (Peeling and Sutherland, 1993, Aasly et al., 1999, Petroff et al., 2002b).

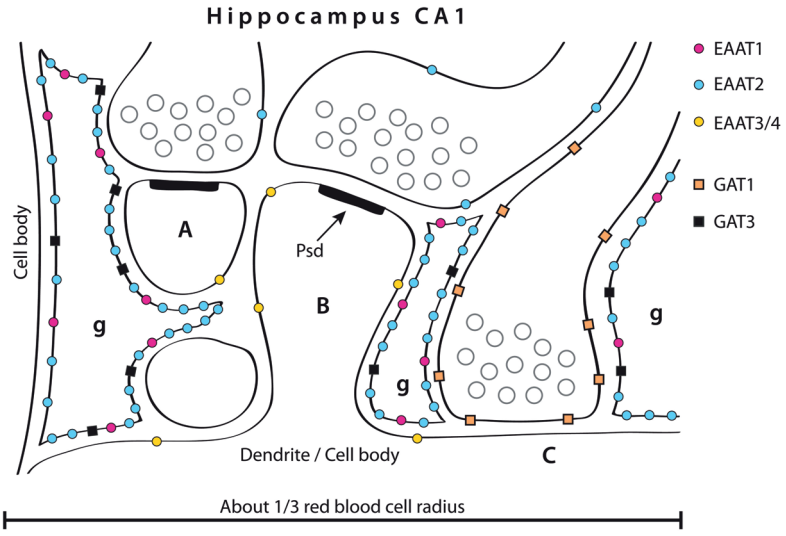

Figure 3.
Distribution of GABA and glutamate transporters in the around a synapse in hippocampus. Fine astrocyte branches (g) are found adjacent to both the glutamatergic (A and B) and GABAergic synapses (C). The post-synaptic densities (PSD) are seen on the two glutamatergic synapses (A and B) with typical synaptic asymmetry. The GABAergic synapses (C) are more symmetric and are often on dendritic trunks rather than spines. Glutamate and GABA transporters are indicated on the neurons and astrocytes. GLAST is selective for astrocytes (Lehre et al., 1995), while GLT-1 is predominantly expressed in astrocytes (Danbolt et al., 1992). Approximately 10% of the GLT-1 is located on the hippocampal nerve terminals (Furness et al., 2008). The glutamate transporter EAAT3 is selective for neurons and is expressed at levels that are around 100 times lower than those of GLT-1 (Holmseth et al., 2012). Figure from (Zhou and Danbolt, 2013) reproduced with permission.

The glutamate-glutamine cycle in epilepsy

After glutamate has acted on receptors, it must be cleared from the synaptic cleft to ensure good signal-to-noise ratios for neurotransmission and to avoid cell damage due to excessive stimulation (excitotoxicity). Glutamate is transformed to glutamine by the glial-specific enzyme glutamine synthetase (GS) inside the astrocyte after uptake (Norenberg and Martinez-Hernandez, 1979, Derouiche and Frotscher, 1991). Then Glutamine is released from the astrocyte into the extracellular space through a system N glutamine transporter (SN) (Chaudhry et al., 2002). Glutamine has no effect on receptors and may be released with no risk of excitotoxicity. Glutamine is then converted back to glutamate by phosphate-activated glutaminase (PAG), which is located in the outer mitochondrial membranes inside the neuron (van den Berg and Garfinkel, 1971, Kvamme et al., 2008). Finally, glutamate enters excitatory vesicles (Fremeau et al., 2004). The glutamate is now recycled and again ready to be released into the synaptic cleft (Pow and Crook, 1996). This process is referred to as the glutamate-glutamine cycle and is important because it allows glutamate to be inactivated by glial cells and transported back to neurons in an inactive (non-toxic) form (Gjessing et al., 1972, Hamberger et al., 1983,

Hamberger and Nystrom, 1984). A study using ^{13}C magnetic resonance spectroscopy of excised tissue from humans suggested that the accumulation and impaired clearance of glutamate could be due to a slowing of the glutamate-glutamine cycle (Petroff et al., 2002a). Further supporting a functional defect in glutamate-glutamine cycling was the finding that the enzyme glutamine synthetase (GS), responsible for converting glutamate to glutamine in astrocytes, was down-regulated in sclerotic hippocampi (Eid et al., 2004a, van der Hel et al., 2005). It is possible that these changes in glutamate cycling may contribute to hyperexcitability and the propensity for chronic seizures, but the role of these changes in epileptogenesis and the relative contributions of astrocytes and neurons in impaired glutamate homeostasis are not clear. The correlation of hippocampal sclerosis and reduced hippocampal volume with elevated extracellular glutamate levels indicates that there is an impaired glutamate uptake capacity in MTLE. One possible cause of this impairment may be a down-regulation of glutamate transporters (Meldrum et al., 1999).

Glutamate transporters in mesial temporal lobe epilepsy

Glutamate is primarily taken up by astrocytes and, is rapidly converted to the non-excitotoxic amino acid glutamine (Broman et al., 2000) by the glial-specific enzyme glutamine synthetase (GS) (Norenberg and Martinez-Hernandez, 1979, Derouiche and Frotscher, 1991). The glutamate uptake system (for a review, see: (Danbolt, 2001) and (Zhou and Danbolt, 2013)) consists of five different transporter proteins and represents the only (significant) mechanism for the removal of glutamate from the extracellular fluid in the brain. Five different glutamate (excitatory amino acid) transporters have been identified: GLAST (EAAT1; slc1a3) (Storck et al., 1992, Tanaka, 1993), GLT-1 (EAAT2; slc1a2) (Pines et al., 1992), EAAT3 (EAAC; slc1a1) (Kanai and Hediger, 1992), EAAT4 (slc1a6) (Fairman et al., 1995), and EAAT5 (slc1a7) (Arriza et al., 1997). GLAST is expressed in astrocytes, while GLT-1 is expressed in both astrocytes and neurons (Danbolt et al., 1992, Levy et al., 1993, Chaudhry et al., 1995, Lehre et al., 1995, Chen et al., 2004, Furness et al., 2008). EAAT3 is neuronal and predominantly postsynaptic (Rothstein et al., 1994, Holmseth et al., 2012). EAAT4 is a neuronal postsynaptic glutamate transporter in Purkinje cell spines (Yamada et al., 1996, Nagao et al., 1997, Dehnes et al., 1998), and EAAT5 is primarily expressed in the retina (Arriza et al., 1997). Several splice variants of the various EAATs have been reported (Utsunomiya-Tate et al., 1997, Meyer et al., 1998, Meyer et al., 1999, Huggett et al., 2000, Rauen et al., 2004, Rozyczka and Engele, 2005). Although both neurons and glia contain glutamate transporters, it is generally accepted that more than 90 % of the forebrain glutamate uptake activity is mediated by GLT-1 (Danbolt et al., 1992, Haugeto et al., 1996, Tanaka et al., 1997) (for a review and discussion, see (Danbolt, 2001) and (Zhou and Danbolt, 2013)). The two main glutamate transporters, GLAST and GLT-1, exhibit complementary expression patterns. GLAST is preferentially expressed in the cerebellum, whereas GLT-1 is more prevalent in the forebrain structures (Lehre et al., 1995). For a more detailed review, see (Zhou and Danbolt, 2013). Previously, it was believed that GLT-1 was expressed exclusively in astroglia in the normal central nervous system (Danbolt et al., 1992, Levy et al., 1993, Rothstein et al., 1994, Chaudhry et al., 1995, Lehre et al., 1995, Schmitt et al., 1996, Ullensvang et al., 1997), with the exception of the retina (Rauen and Kanner, 1994, Rauen et al., 1996, Harada et al., 1998). Later studies show that GLT-1 is expressed in the axon terminals and spines of neurons (Chen

et al., 2004, Berger et al., 2005, Furness et al., 2008, Melone et al., 2009). The splice variant GLT-1a is the predominant neuronal isoform and is expressed in terminals in the hippocampus (Furness et al., 2008) and in the somatic sensory cortex (Melone et al., 2009). The GLT-1a protein accounts for approximately 90 % of all GLT-1 variants (Holmseth et al., 2009).

The expression of the two major high-affinity glutamate transporter proteins, GLT-1 and GLAST, has been investigated in brain tissue from various seizure models and from patients with localization-related epilepsies. Reduced expression of glutamate transporters is shown to cause or be associated with seizures in animal models. A relationship between glutamate transporter dysfunction and epilepsy has been established by Tanaka and colleagues, who reported that mutant mice lacking GLT-1 had increased extracellular glutamate levels and developed spontaneous seizures (Tanaka et al., 1997). Several studies using animal models have linked glutamate transporter expression and epilepsy, but the results of these studies have been contradictory. The majority of studies showed a decrease in GLAST and GLT-1 mRNA and protein (Samuelsson et al., 2000, Ingram et al., 2001, Ueda et al., 2001) and an increase in EAAT3 mRNA and protein (Ghijsen et al., 1999, Ueda et al., 2001, Crino et al., 2002, Gorter et al., 2002) in epileptic animal models. Other studies demonstrated an increase in GLAST mRNA (Nonaka et al., 1998) and a decrease in EAAT3 mRNA (Akbar et al., 1998), while Simantov et al. found a down-regulation of EAAT3 and a modest increase in the expression of GLT-1 in restricted hippocampal regions (Simantov et al., 1999). A deficiency in GLAST has also been shown to increase the duration of generalized seizures in amygdala-kindled rats (Watanabe et al., 1999).

There are some studies reporting on glutamate transporters in human epileptic patients. Tessler and colleagues (1999) found no change in the level of mRNA or protein using in situ hybridization and Western blotting techniques, respectively, for GLAST and GLT-1 in the hippocampus or the temporal cortex in temporal lobe epilepsy (TLE) patients compared with postmortem controls (Tessler et al., 1999). Another study found that the expression of the glutamate transporter GLT-1 was decreased in areas with neuronal loss in the epileptogenic hippocampi formation (Mathern et al., 1999, Proper et al., 2002). Mathern and colleagues used immunocytochemical methods coupled with neuronal counting and image analysis. No changes in GLAST immunoreactivity (IR) were observed in the hippocampi of TLE patients with hippocampal sclerosis compared with TLE patients without hippocampal sclerosis. Decreased GLT-1-IR in the same study was associated with neuronal loss, while increased EAAT3-IR was seen in areas where neurons were spared and a decrease was seen in areas with neuronal loss (Mathern et al., 1999). Proper and colleagues showed a general decrease in GLAST-IR and GLT-1-IR in the sclerotic hippocampus compared with the non-sclerotic hippocampus or postmortem controls (Proper et al., 2002). This decrease was accompanied by a decrease in mRNA levels of both transporters. An increase in neuronal EAAT3 protein levels in the resistant areas (CA2 region, granule cell layer, and subiculum) in the hippocampal sclerosis group was also observed. Other investigators failed to detect alterations in astroglial glutamate transporters in human TLE or in laboratory models of seizures. For example, in patients

undergoing resective brain surgery for the treatment of TLE, no differences in the expression of GLT-1 or GLAST were found.

GABA transporters in mesial temporal lobe epilepsy

Inhibition of GABA transport increases the extracellular levels of GABA and mediates seizure protection (Nielsen et al., 1991); the therapeutic potential of GABA transporters (GAT) inhibition has been confirmed with the successful development of the GAT1 selective drug tiagabine (Nielsen et al., 1991). An increase in GABAergic interneurons expressing GAT1 has previously been reported in the rat neocortex 24 hours after corticotropin-releasing hormone-induced seizures (Orozco-Suarez et al., 2000), and a transient increase in GAT3 mRNA (but not protein) expression has been described in amygdala-kindled rats 1 hour after the last seizure (Hirao et al., 1998). These findings suggest that seizure activity is associated with an upregulation of neocortical GAT expression. Increased GAT expression could lower extracellular GABA levels, thus contributing to the origin and spread of epileptic activity. Alternatively, because GABA transporters can function in reverse (Sihra and Nicholls, 1987, Pin and Bockaert, 1989, Attwell et al., 1993, Levi and Raiteri, 1993, Jaffe and Figueroa, 2001, Wu et al., 2006), an up-regulation of GATs following sustained neuronal activity may induce a compensatory increase in GABA release via a non-vesicular, transporter-mediated mechanism (During and Spencer, 1993, Gaspary et al., 1998, Wu et al., 2006). Transgenic mice that overexpress the GAT1 protein exhibit increased susceptibility to chemically induced seizures, although they do not display spontaneous seizure activity (Ma et al., 2001). Enhanced GAT1-mediated GABA transport was also associated with seizures in a genetically epileptic mouse strain (Fueta et al., 2003). Evidence for a down-regulation of GAT function in the epileptic neocortex has also been reported. Cortical GABA uptake is reduced in different genetic mouse and rat models of epilepsy (Cordero et al., 1994, Cordero et al., 1994b, Sutch et al., 1999), and GAT1 mRNA expression is reduced in the neocortex of genetically epileptic-prone rats (Akbar et al., 1998). In a pilocarpine rat model of TLE, GAT1 was found to be reduced, particularly in the perisomatic axon terminals in the sensorimotor cortex (Silva et al., 2002). Hippocampal GABA transporter immunoreactivity differs in TLE patients compared with autopsy tissue (Mathern et al., 1999). There was an increase in GAT1 expression in the outer molecular layer of the hippocampal dentate and increased astrocytic GAT3 in the hilus and Ammon's horn. In contrast, another study showed that GAT1 was reduced and abnormally distributed in the neocortex of patients with TLE and focal dysplasia (Spreafico et al., 2000). The substantial reduction in perisomatic terminals expressing GAT1 reported in the epileptic neocortex (Silva et al., 2002) and hippocampus (Sayin et al., 2003, Arellano et al., 2004) is in line with the notions that the loss of the GABAergic cells innervating the soma and initial axon segment of pyramidal neurons, i.e., the basket and chandelier cells, is crucial to seizure onset (Ribak et al., 1978, Ribak et al., 1979, Sloper et al., 1980, Sloviter, 1987, DeFelipe, 1999), but that the reduction of GABA transport and GAT expression may represent a compensatory response to homeostatically modulate neuronal overexcitation (Soudijn and van Wijngaarden, 2000). Moreover, reduced GAT expression in epileptic tissue may exacerbate epileptiform activity by decreasing the scope of GABA heterotransport (During et al., 1995, Gaspary et al., 1998, Patrylo et al., 2001).

Materials and Methods

Human tissue

A challenge in studies of resected human epileptogenic tissue is to obtain appropriate controls. The study of human MTLE hippocampal formation included in this thesis is based on surgically resected material from patients with epilepsy with a similarly resected MaTLE and PTLE hippocampal formation as controls. This control tissue is superior to autopsy material because the control tissue has been resected in the same manner as the tissue from MTLE patients. Moreover, the control and MTLE tissues have been subjected to similar regimens of antiepileptic drugs and have suffered a comparable degree of seizure activity. It is important to control for these factors because each factor may cause structural and/or biochemical changes capable of interfering with the interpretation of results. Additionally, unlike autopsy tissue, the surgically resected control tissue has not been subjected to proteolysis or other changes typical of postmortem tissue (Beckstrøm et al., Tessler et al., 1999, Zhou et al., 2012). The use of human tissue for these studies was approved by the Human Investigative Committee at Yale University.

Studies of human epileptogenic hippocampi

Patients with medically intractable TLE underwent phased presurgical evaluation at the Yale-New Haven Hospital; appropriate candidates underwent an anteromedial temporal lobectomy with hippocampectomy according to standard procedures (Spencer et al., 1984). Informed consent from each patient and institutional approval were obtained for the use of tissue in this project. The surgically resected hippocampi were classified into two groups, namely, (1) mesial temporal lobe epilepsy (MTLE) and (2) non-MTLE, as described by Eid et al. (Eid et al., 2004). The MTLE hippocampus was characterized by pronounced neuronal loss (>50 %) and extensive astroglial proliferation that was most pronounced in the hippocampal subfields of the CA1, CA3, and hilus regions (Kim, 2001). Reorganization of peptidergic neurons (neurons containing dynorphin, somatostatin, neuropeptide Y, and substance P) in the dentate gyrus were also seen (de Lanerolle et al., 1994). In contrast, the non-MTLE hippocampi were recognized by a modest (<25 %) neuronal loss throughout all hippocampal subfields, minimal astroglial proliferation, and no reorganization of peptidergic neurons in the dentate gyrus (Kim, 2001). They were obtained from patients with a mass lesion in the temporal lobe outside the hippocampus (MaTLE) and those with no clear etiology, described previously as paradoxical temporal lobe epilepsy (PTLE) (de Lanerolle et al., 1997).

Tissue preparation

Immediately after surgical resection, two 5-mm-thick slices were cut from the mid-portion of the hippocampus. One of the samples was immersed in a fixative containing 4 % formaldehyde and 15 % (v/v) of a saturated picric acid solution in 0.1 M phosphate buffer (PB), pH 7.4, for 1 hour, followed by immersion in 5 % acrolein in PB for 3 hours. Thereafter, the tissue was rinsed and stored in PB at 4 °C. Fifty-micrometer-thick coronal sections were cut on a vibratome and processed for immunohistochemistry. The other

sample was rapidly frozen on dry ice, stored at –80 °C, and later used for Western blot analysis.

Hippocampal nomenclature

The mesial temporal lobe consists of the hippocampus, parahippocampal gyrus, entorhinal cortex, and amygdala. The hippocampus is commonly divided into the subiculum, Ammon's horn, and dentate gyrus (Lorente de Nó, 1934). The Ammon's horn is further subdivided into the CA1, CA2, and CA3 regions, while the dentate gyrus is subdivided into the hilus, granule cell layer, and molecular layer (Amaral and Witter, 1989).

Antibodies

Peptides

Peptides representing parts of the glutamate and GABA transporter amino acid residues in the sequence were used in the antibody production. Map-peptides were used for immunization without coupling to carrier proteins, while the other peptides were coupled to either KLH, rabbit serum albumin, or thyroglobulin with either glutaraldehyde (with or without reduction with sodium borohydride) or MBS, as described previously (Danbolt et al., 1998). Antigenicity profiles were calculated for rat GLT-1 and EAAT3 according to Jameson and Wolf (Jameson and Wolf, 1988) using the Protean program (DNASTAR, Inc., Madison, WI, USA). The animals were immunized with peptides and bled as described previously (Danbolt et al., 1998).

Antisera

In the first paper, antibodies against GAT1 (slc6a1) and GAT3 (slc6a11) were raised in rabbits and purified. The GAT1 antibodies were directed against amino acids 584-599 in the rat sequence (GPEQPQAGSSASKEAYI), which only differs by three amino acids in humans (GPEHAQAGSSTSKEAYI). The GAT3 antibody was raised against the C-terminus (amino acids 607-677). Both antibodies showed high specificity in immunoblots of human hippocampal tissue.

In Paper 2, antibodies against GLAST and GLT-1 were prepared by immunizing rabbits with synthetic peptides (A522-541 (PYQLIAQDNEPEKPVADSET) for GLAST and B563-573 (SVEEEPWKREK) for GLT-1) coupled to keyhole limpet hemocyanin with glutaraldehyde (Lehre et al., 1995, Danbolt et al., 1998). The corresponding anti-peptide antibodies are referred to as anti-A522 (rabbit 8D0161; Ab#314) and anti-B563 (rabbit 1B0707; Ab#355). These antibodies were also tested and characterized by Western blotting and immunohistochemical methods and cross-reacted with human GLAST and GLT-1, respectively.

Paper 4, which focuses on antibody specificity, showed that antibodies against the peptides could be isolated from the antisera in most cases, albeit in highly variable amounts (0-300 µg/ml serum). Testing of crude antisera was usually performed, but relevant antibodies were affinity-purified, as described previously, on columns containing

covalently immobilized antigen (Lehre et al., 1995, Danbolt et al., 1998). Sera from rabbits immunized with multiple peptides were passed through one affinity column for each of the peptides used for immunization. This procedure resulted in different antibody fractions, which were named according to the antigen immobilized on the respective affinity column. In this paper, the antiserum derived from rabbits was subjected to a four-stage purification process, which included absorption against tubulin. Purified antibodies were spectrophotometrically quantified at 280 nm using bovine IgG as the standard.

ELISA procedure for antibody testing

ELISA, or enzyme-linked immunosorbent assay is a common laboratory technique that we used to test antibody specificity. In the ELISA procedure the separation of specific and non-specific interactions occurs via serial binding to a solid surface, usually a polystyrene multiwell plate (microtiterplate). The steps of the ELISA usually result in a colored end product which correlates to the amount of antigen present in the original sample, but we were, on the other hand, interested to see if the antibody binds to the antigen or not. ELISAs are quick and simple to carry out and, because it was performed by a pipetting robot (Tecan Genesis 200 Workstation), it was possible to test multiple antibodies simultaneously. The microtiterplates were coated with a known amount of antigen and antibodies were tested in different concentrations. Briefely; each well in the 96-well microtiterplate was first incubated (2 hours) with 50 µl TBS (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05 % NaN_3) containing 3 µg antigen per ml and then washed with TBS (4 cycles, 50 s) to remove unbound antigen. To block free binding sites, the wells were incubated with TBS (380 µl/well) containing 20 % NCS for 2 hours with agitation and washed in TBS with 0.05 % (v/v) Tween 20 (TBST) (4 cycles, 50 s). Antibody fractions to be tested were diluted in blocking solution to the desired concentration. A 50-µl sample was added to each well, incubated for 60 min, and washed with TBST (8 cycles, 50 s). The wells were incubated for 60 min with 50 µl of TBST containing 20 % NCS and alkaline phosphatase-conjugated anti-rabbit diluted 1:1000. A final washing with TBST (8 cycles, 50 s) was followed by the addition of 100 µl of p-nitrophenyl phosphate (1 mg/ml) in 0.1 M diethanolamine-HCl buffer (pH 9.8) with 1 mM $MgCl_2$. The OD405 was measured after 60 min. Background levels in each assay were determined using BSA as the coating antigen.

Immunohistochemistry

Immunohistochemistry refers to a technique where you the detect antigens, in our case transporter proteins, in tissue sections by exploiting the principle of antibodies binding specifically to the antigens of interest. Immunohistochemisty followed by microscopy was useful for studying the transporter protein's localization at the regional, cellular, and subcellular levels. Vibratome sections in human hippocampal tissue were blocked and incubated free-floating in anti-A522 (200 ng/ml) or anti-B563 (200 ng/ml) antibodies for 24 hours (RT), and they were then processed according to the avidin-biotin peroxidase method (Hsu et al., 1981) using a commercially available kit (Vectastain Elite, Vector Laboratories, Burlingame, CA). The immunoreagents diffused into the sections from the surface, and the sections from each patient were mounted on slides, dehydrated, and cover-slipped for light microscopy analysis. Control sections incubated without the

primary antibody or by replacing the primary antibody with preimmune sera were not immunostained.

Electron microscopy

An electron microscope (EM) is a microscope that utilize the same principles as an optical microscope, but rather than visible light, uses accelerated electrons as a source of illumination. Because the wavelength of an electron is much shorter than that of visible light photons, the electron microscope has a higher resolving power than a light microscope and can reveal the structure of smaller objects. Electron microscopes are used to investigate the ultrastructure of a wide range of specimens and we used EM to evaluate transporter protein in relation to cell types and cellular structures. All electron microscopy samples were prepared by labeling and resin embedding before placed in the microscope vacuum. After immunostaining as described above, 45-70 nm thin sections were treated with 2 % osmium tetroxide in PB, stained en bloc with 2 % uranyl acetate in water, dehydrated, and flat-embedded in a plastic resin (Durcupan ACM). Sections were cut on an ultramicrotome, transferred to 500-mesh copper grids (Electron Microscopic Sciences, Fort Washington, PA, USA), contrast-stained with lead citrate, and examined by a transmission electron microscope [Jeol 1200 EX II] at 60 kV.

Western blot analysis

Western blotting is a widely used analytical technique used to detect specific proteins in a sample of tissue homogenate or extract. The first step in a Western blotting procedure is to separate the tissue proteins by their size using gel electrophoresis and then transfer the proteins onto a nitrocellulose or polyvinylidene difluoride (PVDF) membrane. Next, the membrane is blocked to prevent any nonspecific binding of antibodies to the surface of the membrane and the transferred protein of interest is complexed with an enzyme-labeled antibody as a probe. An appropriate substrate is then added to the enzyme and together they produce a detectable product (e.g. colorimetric detection). Proteins in a tissue homogenate from human hippocampi were separated by molecular weight by SDS-PAGE (Laemmli, 1970) and subsequently transferred from the gel to a membrane (Towbin et al., 1979). The proteins on the nitrocellulose membranes were immunolabeled with the antibodies specific for the glutamate and GABA transporters. Secondary antibodies conjugated to alkaline phosphatase or horseradish peroxidase enabled visualization of probed antigens, and the immunocomplexes were detected by chemoluminescence, which is a very sensitive method. A standard curve was created with increasing concentrations of homogenized hippocampal tissue from a non-MTLE patient to determine the linear range. A CCD camera captured the chemoluminescence signals. We used a Kodak camera, which converts optical brightness into electrical amplitude using a charge-coupled device (CCD). The signals were converted to digital values using Kodak software. The CCD camera we used from Kodak provided a digital recording after very short exposure times. Both antibodies yielded a strong, single band consistent with the expected molecular mass of the glial glutamate transporters. Substitution of the primary antibodies with normal serum completely abolished the staining. The blots were assessed using a two-tailed Mann-Whitney U test (Statview Software, SAS Institute, NC).

Mouse tissue

All animal experimentations were carried out in accordance with 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 the Norwegian Governmental Institute of Public Health (Oslo, Norway). Care was taken to minimize the number of animals used and to avoid suffering. Mice were housed and bred as described previously (Zhou et al., 2012). Mice lacking GLT-1 were originally generated by Tanaka (Tanaka et al., 1997), and the breeding of heterozygous mice in a C57Bl/6 background produced the knockout and wild-type mice. All experiments were performed using the wild-type and knockout littermate pairs to maximize the probability that differences between wild-type and knockout mice were due to GLT-1 and not to other factors, such as age or rearing conditions. Because the GLT-1 knockout mice tended to die prematurely (Tanaka et al., 1997), the mice used were approximately 3 weeks old. At this age, GLT-1 and GLAST expression levels are approximately half of the adult levels (Ullensvang et al., 1997, Diamond, 2005).

Animal treatment and tissue collection

In Paper 3, five GLT-1 knockout and seven wild-type mice were administered an intraperitoneal injection with 70-150 µl of 0.3 M [1-^{13}C]glucose (543 mg/kg) plus 0.6 M [1,2-^{13}C]acetate (504 mg/kg). Fifteen minutes later, the mice were decapitated, and their heads were immediately frozen in liquid nitrogen and stored at -80 °C. The tissue samples were subjected to a water/methanol-chloroform extraction method as previously described (Le Belle et al., 2002). Samples were homogenized in 300 µl of methanol using a Vibra Cell™ sonicator (Model VCX 750, Sonics & Materials, Newtown, CT, USA), and α-ABA was used as an internal standard for HPLC analysis. After extraction, samples were lyophilized and re-suspended in 200 µl of deuterium oxide (D2O); 10 µl was removed for HPLC analysis, and the rest was lyophilized and re-dissolved in 120 µl of D2O containing 0.2908 g/l TSP and 0.1 % ethylene glycol as internal standards for quantification in ^{13}C and ^{1}H NMR analysis.

High-performance liquid chromatography (HPLC)

The samples were analyzed using a Hewlett Packard 1100 System (Agilent Technologies, Palo Alto, CA, USA) with fluorescence detection after derivatization with o-phtaldialdehyde (Geddes and Wood). The amino acid components were separated with a ZORBAX SB-C18 (4.6 × 150 mm, 3.55 µm) column from Agilent using 50 mM sodium phosphate buffer (pH 5.9) with 2.5 % tetrahydrofurane plus methanol (98.75 %) and tetrahydrofurane (1.25 %) as eluents.

^{13}C and ^{1}H magnetic resonance spectroscopy

Nuclear magnetic resonance (NMR) is better known as magnetic resonance imaging (MRI) or magnetic resonance spectroscopy (MRS) in clinical practice. The main principle of NMR is based on the fact that nuclei with an uneven mass number and/or an odd number of protons, such as ^{1}H and ^{13}C, have magnetic properties (nuclear spin).

In ^{13}C magnetic resonance spectroscopy (^{13}C MRS) experiments with a ^{13}C-labeled substrate, one exploits the fact that most carbon atoms are ^{12}C (without a magnetic moment) and only 1.1 % are ^{13}C (with a magnetic moment). Thus, by in vivo administration of ^{13}C-labeled substrates such as [1-^{13}C]glucose and 1,2-^{13}C]acetate used in this thesis, followed by ^{13}C and ^{1}H MRS of brain extracts, the incorporation and exact position of ^{13}C in important metabolites can be detected. Specific peaks in the spectra that are produced represent different compounds in the sample. The amount of ^{13}C in different metabolites is quantified from integrals of the relevant peaks in the spectra using ethylene glycol as an internal standard with a known amount of ^{13}C. Furthermore, ^{13}C MRS allows the study of metabolic interactions between different cellular compartments (Sonnewald and Kondziella, 2003). The spatial segregation of the enzymes PAG, GAD, and GS to different cell types causes the majority of glutamate, GABA, and glutamine to be synthesized in different compartments. The glutamatergic neurons contain the largest pool of glutamate, whereas GABAergic neurons hold the GABA pool (Storm-Mathisen et al., 1983, Ottersen and Storm-Mathisen, 1985). Both types of neurons contain PAG (Kvamme et al., 1988), whereas only astrocytes contain GS (Martinez-Hernandez et al., 1977). The interpretation of the MRS data is further based on the knowledge of the metabolic fate of [1-^{13}C]glucose and [1,2-^{13}C]acetate in neurons and astrocytes. Glucose is taken up into both neurons and astrocytes, but it is metabolized to a greater extent in neurons (Qu et al., 2000b). However, acetate is almost exclusively metabolized in astrocytes (Hassel and Sonnewald, 1995a, Waniewski and Martin, 1998). Thus, administrating both [1-^{13}C]glucose and [1,2-^{13}C]acetate to animals enables a more detailed metabolic analysis of the different cellular compartments.

Analysis of all samples in Paper 3 was performed using a QCI CryoProbe™ 600-MHz ultrashielded Plus magnet (Bruker BioSpin GmbH, Reinstetten, Germany). ^{1}H NMR spectroscopy was employed to quantify the amount of amino acids and lactate in extracts from the frontal cortex and cerebellum of wild-type and GLT-1 KO mice. ^{1}H NMR spectra were acquired using a pulse angle of 90°, a 12-kHz spectral width and derived from 66 data points, an acquisition time of 2.66 s, a relaxation delay of 10 s, and 128 scans. Proton-decoupled ^{13}C MR spectra were obtained on the same instrument using a 30° pulse angle, a 30-kHz spectral width derived from 98,000 data points, an acquisition time of 1.65 s, and a relaxation delay of 0.5 s. The number of scans needed to obtain an appropriate signal-to-noise ratio was 10,000. TopSpin™ 3.0 software (Bruker BioSpin GmbH, Reinstetten, Germany) was used for the acquisition, integration, and quantification of data.

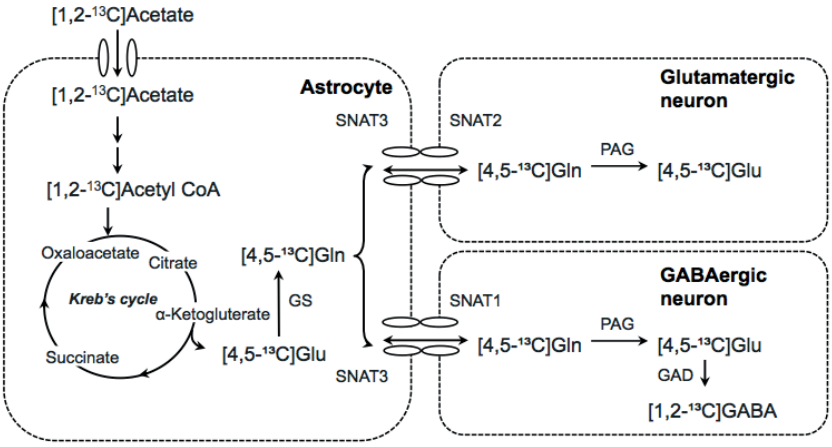

Figure 4.
Schematic presentation of glial interactions with glutamatergic and GABAergic neurons following the uptake of [1,2-^{13}C]acetate. [1,2-^{13}C]acetate is preferentially metabolized in astrocytes and enters astrocytic TCA (Kreb's cycle) as [1,2-^{13}C]acetyl CoA. [4,5-^{13}C]α-ketoglutarate may leave the astrocytic TCA cycle as [4,5-^{13}C]glutamate, which can then be transformed to [4,5-^{13}C]glutamine by the action of the astrocyte-specific enzyme glutamine synthetase. [4,5-^{13}C]glutamine then leaves astrocytes via glutamine transporters (SNAT3) into the extracellular compartment from where it is taken up by neurons (SNAT1 in GABAergic neurons and SNAT2 in glutamatergic neurons). [4,5-^{13}C]glutamine may be oxidized as an energy source or alternately deamidated to [4,5-^{13}C]glutamate by the action of phosphate-activated glutaminase; it may also be a substrate of glutamate decarboxylate (GAD) in the formation of [1,2-^{13}C]GABA in neuronal cell bodies. To date, glutamine transporters have not been directly identified in glutamatergic terminals. Abbreviations: GAD: glutamate dehydrogenase; Glu: glutamate; Gln: glutamine; GS: glutamine synthetase; PAG: phosphate-activated glutaminase. (Bjørnsen et al., 2014)

Data analysis and interpretation in paper 3

To understand the labeling pattern of metabolites after the administration of the labeled substrates [1-^{13}C]glucose and [1,2-^{13}C]acetate, it is necessary to consider their major biochemical pathways. Glucose is taken up and utilized by both neurons and astrocytes; however, 66 % of acetylCoA produced from glucose in the brain enters the neuronal tricarboxylic acid cycle (TCA) (Hassel and Sonnewald, 1995a, Qu et al., 2000a). Following uptake, [1-^{13}C]glucose enters glycolysis, which results in the formation of one molecule of [3-^{13}C]pyruvate and one molecule of unlabeled pyruvate. [3-^{13}C]pyruvate can be converted to [3-^{13}C]lactate or [3-^{13}C]alanine in the cytosol or enter the mitochondria and be converted to [2-^{13}C]acetylCoA. [2-^{13}C]acetylCoA condenses with oxaloacetate to form the TCA cycle intermediates [2-^{13}C]citrate and [4-^{13}C]α-ketoglutarate. The ^{13}C label can exit the TCA cycle in the form of [4-^{13}C]α-ketoglutarate, which can be converted to [4-^{13}C]glutamate or alternately continue in the cycle. The [4-^{13}C]glutamate, which is either taken up by astrocytes or made by astrocytes, can be converted to [4-^{13}C]glutamine by the enzyme glutamine synthetase (GS), or it can enter the TCA cycle in astrocytes after conversion to [4-^{13}C]α-ketoglutarate to ultimately form [3 or 2-^{13}C]glutamine. Any glutamine taken up by neurons may be converted back to glutamate by phosphate-activated glutaminase (PAG) in the mitochondria. However, if

the ^{13}C label from [1-^{13}C]glucose remains in the TCA cycle for a second round, equal amounts of ^{13}C will be incorporated into the 2 or 3 positions of glutamate and glutamine (Melo et al., 2006). Unlike glucose, acetate is taken up only by astrocytes (Waniewski and Martin, 1998). After uptake by astrocytes, [1,2-^{13}C]acetate is converted to [1,2-^{13}C]acetylCoA, which then condenses with oxaloacetate to form TCA cycle intermediates. [4,5-^{13}C]α-ketoglutarate can be converted to [4,5-^{13}C]glutamate, which is rapidly converted to [4,5-^{13}C]glutamine by the astrocytic GS. Glutamine can then be transported to neurons and converted back to [4,5-^{13}C]glutamate via phosphate-activated glutaminase.

Relevant peaks in the spectra were assigned and quantified from the integrals of the peaks using ethylene glycol as an internal standard with known amounts of ^{13}C. Corrections for natural abundance as well as nuclear Overhauser enhancement and relaxation effects relative to the internal standard were applied to all relevant integrals from ^{13}C spectra.

Statistics

Student's t-tests were used, and $p < 0.05$ was regarded as significant.

Summary of individual papers

Paper 1

"GAT1 and GAT3 expression are differently localized in the human epileptogenic hippocampus"

Background

The involvement of GABA in TLE is controversial and the distribution of GABA transporters in the human hippocampus has been described in only two other studies (Mathern et al., 1999, Arellano et al., 2004b). It has been hypothesized that the diminished GABA release is caused by a reduction of GABA transporters (During et al., 1995), although no direct measurements of GABA transporters in the human hippocampus have been performed. Previous studies have only studied immunoreactivity on tissue sections, so we used immunohistochemistry and immunoblots. In this paper, we evaluated whether the low levels of glutamate-induced extracellular GABA in the epileptogenic hippocampus (During and Spencer, 1993) were related to changes in the expression of the GABA transporters GAT1 and GAT3.

Results

The GABA transporters were localized by immunohistochemistry in hippocampi removed from patients with medically intractable TLE. The antibodies specificity were tested on immunoblots, but not checked against knockout tissue. The GAT3 showed an additional single narrow band with molecular weight lower than expected for the transporter protein. GAT1 was localized in axon terminals and small neuronal cell bodies and showed reduced immunoreactivity in the dentate granule cells, CA1 and CA3 regions of the epileptogenic hippocampus. GAT3 was weakly expressed on astrocytes in non-sclerotic hippocampi, but was more prominently expressed in sclerotic hippocampi. GAT3 expression was confined to cells resembling protoplasmic astrocytes, which were located in regions of relative neuronal sparing, such as the dentate gyrus and hilus of the sclerotic hippocampus. Despite these changes in immunoreactivity, Western blot analysis showed no significant difference in GAT1 or GAT3 levels between the two groups. However, immunohistochemical staining showed regional differences in GAT1 immunoreactivity within the hippocampus.

Discussion

Taken together with Western blot data, these findings may reflect a compensatory up-regulation and may account for the lack of the difference between sclerotic and non-sclerotic hippocampi in the immunoblots. It may be inferred from this paper that rather than an overall decrease in GABA transporters, there is, at best, no overall change. Theoretically, the local GAT1 reductions found in our study may have caused regional increases in the excitability of neurons, e.g., the dentate granule cells, because of the loss of inhibition normally associated with GABA release from GAT1-bearing terminals. The increase in GAT3 astrocytes in TLE may also contribute to the excitable state by excess removal of GABA from the extracellular space. It should be noted that the antibodies specificity were not tested on knockout mice.

Paper 2

"Changes in glial glutamate transporters in human epileptogenic hippocampus: inadequate explanation for high extracellular glutamate during seizures"

Background

Elevated levels of extracellular glutamate have been observed in patients with various epilepsies (Janjua et al., 1992, Ferrie et al., 1999) and have also been reported to be elevated in epileptogenic hippocampi. Glutamate is thought to play a major role in the initiation and spread of seizure activity (Meldrum, 1994) and intracellular recordings from human MTLE hippocampi reveal a glutamate-dependent hyperexcitability (Williamson and Spencer, 1994). Previous studies of the distribution of GLAST and GLT-1 in surgically removed human sclerotic hippocampi compared with that in non-sclerotic hippocampi concluded that there is a reduction in GLT-1 (Mathern et al., 1999, Proper et al., 2002), a slight or no reduction in GLAST (Mathern et al., 1999), or essentially no change in these transporters (Tessler et al., 1999). The aim of this study was to further explore the differences in glial transporter distribution in the non-sclerotic (non-MTLE) and sclerotic (MTLE) hippocampi of TLE patients.

Results

Western blotting was performed on human tissue that was obtain from the OR. There were no overall significant difference in the expression in transporters GLAST and GLT-1 between the non-MTLE and MTLE hippocampi, but the pattern of immunolabeling were different.

Discussion

The previous reports of reduced astrocyte glutamate transporters in the sclerotic hippocampi from patients with TLE are not compelling, but our findings are consistent with Tessler and colleagues (Tessler et al., 1999). Our study used other antibodies than the studies that found a downregulation of GLT-1 and GLAST (Mathern et al., 1999, Proper et al., 2002), which could explain the discrepancies. Both the use of fixatives (Danbolt, 2001) and postmortem autolytic changes (Beckstrøm et al., 1999) may also cause different results. We found it difficult to explain the reduced immunoreactivity in areas such as the CA1 and hilus regions in sclerotic hippocampi, while immunoblot analyses showed no overall difference. Our result may reflect redistribution of the transporter proteins throughout the astrocytic processes in the sclerotic hippocampi, rather than a true reduction in level. Ultrastructural localization studies show GLT-1 immunoreactivity throughout the astrocytic process despite the apparent loss suggested by light microscopy analysis. Likewise, confocal immunohistochemical studies reveal GLT-1 immunoreactivity throughout astrocyte processes, even though staining in somata is reduced (Eid et al., 2004). An alternative possibility is that there may be compensatory changes in the expression of transporters in different regions of the hippocampus. We found little support for glutamate transporters as the direct cause of elevated levels of extracellular glutamate in the epileptogenic hippocampi. Our study cannot exclude the possibility that there may be small changes in transporters, such as splice variants, which are not distinguished by the antibodies used in this study.

Paper 3

"The GLT-1 (EAAT2; slc1a2) glutamate transporter is essential for glutamate homeostasis in the neocortex of the mouse"

Background

Based on the reported correlation between glutamate transporter dysfunction and epilepsy in mutant mice lacking GLT-1 (Tanaka et al., 1997), we wanted to evaluate the role of GLT-1 in glutamate homeostasis. Knockout mice have increased extracellular glutamate levels and developed spontaneous seizures. We know that there is no glutamate uptake activity in hippocampal terminals in GLT-1 knockout mice and our aims were to analyze if the distribution of metabolic substrate between the cellular compartments would be different in these animals.

Results

Using ^{13}C magnetic resonance spectroscopy (MRS) of cortical tissue from mice lacking the glutamate transporter GLT-1 (EAAT2; slc1a2) was performed following an intraperitoneal injection of ^{13}C-labeled glucose and acetate. GLT-1 is expressed at the highest levels in the forebrain (Lehre et al., 1995), and the deletion of the GLT-1 gene causes a reduction in glutamate uptake activity by approximately 95 % (Tanaka et al., 1997, Otis and Kavanaugh, Matsugami et al., 2006, Furness et al., 2008, Kiryk et al., 2008, Holmseth et al., 2009). Metabolite levels were analyzed in extracts from the neocortex and cerebellum and by ^{13}C labeling in the neocortex. Compared with wild-type mice, GLT-1 knockout mice had normal levels of glutamate and glutamine in the cerebellum, but decreased levels in the neocortex. However, ^{13}C-labeled glutamate showed no significant difference in the neocortex, even though a trend was visible. In contrast, a reduction in ^{13}C-labeled glutamine in the GLT-1 knockout mice was present. It is believed that the TCA cycle metabolism of [1-^{13}C]glucose-derived metabolites primarily reflects neuronal energy metabolism (Hassel et al., 1995, Qu et al., 2000a). The contribution of pyruvate carboxylation toward the labeling of these metabolites was unchanged between the wild-type and GLT-1 knockout mice. Labeling from [1,2-^{13}C]acetate was decreased in glutamate and glutamine, indicating reduced mitochondrial metabolism in the astrocytes.

Discussion

These findings suggest that GLT-1 in nerve terminals in the cerebral cortex may significantly contribute to the replenishment of the pool of glutamate transmitter. Thus, the efficiency of the glutamate-glutamine cycle in the replenishment of the glutamate neurotransmitter pool needs reconsideration. Based on the fact that the knockout mice survive the first month of life (Tanaka et al., 1997), it is possible that other glutamate transporters, to a certain degree, are able to prevent initial accumulation of glutamate extracellularly. Our study is not able to determine if other factors, e.g. changes in receptor expression or function, play an important role in neurotoxic protection. We will neither be able to measure the concentration of metabolites in the different intra- and extracellular compartments.

Paper 4

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

Background

Initially, the aim of this study was to quantify and localize EAAT3 to gain insight into its physiological functions and localization. Our group had seen a mismatch between western blotting and labeling of tissue sections when using our EAAT3 antibodies. Data in the literature claimed that EAAT3 was responsible for more than 30% of the total glutamate uptake activity (Rothstein et al., 1996) and we wondered whether this result was correct. Another study supported this belief by Kugler and Schmitt showed strong labeling with colocalization of EAAT3 and tubulin (Kugler and Schmitt, 1999).

Results

During the development of antibodies against the similar peptide (C480-499) that was used by Kugler and Schmitt, we found that there was cross-reactivity with tubulin despite an absence of primary sequence similarly with EAAT3. This observation made us question if the previous studies had used antibodies that recognized multiple proteins. The majority of the antibodies we generated against different peptides did not recognize the native protein, but antibodies raised against the C-terminus of the EAAT3 protein molecule gave rise to weak labeling in Western blots, but remarkably strong labeling in tissue sections. I programed a robotic ELISA-system, which was used to screen antibodies' reactivity toward a number of non-EAAT3 proteins. The antibodies reacted strongly with tubulin and did so with a remarkable specificity. The antiserum was then fractionated. We obtained one fraction that recognized both tubulin and EAAT3 and another fraction that appeared specific for EAAT3. The EAAT3-specific fraction did not label axons. The labeling appeared restricted to the dendrosomatic compartment and there was no labeling of oligodendrocytes or astroglia.

Discussion

Determination of the antibody specificity is not straightforward and might give unexpected results. EAAT3 was thought to be localized in oligodendrocytes, because of the cross-reactivity between EAAT3 and tubulin observed by Kugler and Schmitt. We made similar antibodies that recognized a strong band that did not correspond to the glutamate transporter. Initially, it was thought that this band could represent a novel and much more abundant variant of EAAT3, but it was eventually found, after extensive testing using a robotic ELISA procedure, to correspond to tubulin. This work shows that the cross-reaction with apparently completely unrelated proteins is very common and, importantly, this study also suggests that the pre-adsorption test has little value when testing affinity-purified antibodies. Optimally all antibodies should be tested on knockout tissue to verify specificity.

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