In search for peripheral markers for epilepsy and ALS

- focus on glutamatergic signaling in blood cells -

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- focus on glutamatergic signaling in blood cells -

Op zoek naar perifere markers voor epilepsie en ALS

-gefocust op glutamaterge signaal transductie in bloed cellen-

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
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door

Willemina Minke Bos

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Promotores:
Prof. Dr. O. Van Nieuwenhuizen
Prof. Dr. J.P.H. Burbach

Co-promotor:
Dr P.N.E. de Graan

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"*If you cannot do great things, do small things in a great way*"

Napoleon Hill

"The role of the infinitely small in nature is infinitely large"

Louis Pasteur

Voor mijn ouders

Table of Contents

Abbreviations

Aβ	=	amyloid β-peptide
ACh	=	Acetylcholine
AED	=	anti-epileptic drug
ALS	=	amyotrophic lateral sclerosis
AMPA	=	α-amino-3-hydroxy-5-methyl-isoxazole-4-proprionate
APP	=	amyloid β precursor protein
CA1-4	=	cornu Ammonis 1-4
DG	=	dentate gyrus
EAAC	=	excitatory amino acid carrier
EAAT	=	excitatory amino acid transporter
GABA	=	γ-aminobutyric acid
GLAST	=	glutamate aspartate transporter
GLT	=	glutamate transporter
GluR	=	glutamate receptor
Glu	=	glutamate
GS	=	glutamine synthetase
GTRAP	=	glutamate transporter associated protein
HS	=	hippocampal sclerosis
IR	=	immunoreactivity
JME	=	juvenile myoclonic epilepsy
KA	=	kainic acid
mGluR	=	metabotropic glutamate receptor
MEG	=	magnetoencephalograms
MRI	=	magnetic resonance imaging
MTLE	=	mesial temporal lobe epilepsy
NMDA	=	N-methyl-D-aspartate
NMR	=	nuclear magnetic resonance
PAG	=	phosphate activated glutaminase
PET	=	positron emission tomography
PML	=	polymorphic layer
SE	=	status epilepticus
SGL	=	supragranular layer
SOD	=	superoxide dismutase
SRS	=	spontaneous recurrent seizures
TLE	=	temporal lobe epilepsy
VGLUT	=	vesicular glutamate transporter

Contents of the Introduction

1 Introduction

1 Glutamate neurotransmission

1.1 Glutamate receptors

The amino acid glutamate mediates most of the excitatory synaptic neurotransmission in the brain. It is estimated that over half of all brain synapses release glutamate. After glutamate is released in the synaptic cleft, glutamate can activate both ionotropic and metabotropic receptors. There are three classes of ionotropic glutamatergic receptors named after their selective agonists; N-methyl-D-aspartate (NMDA), α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) and kainate (KA) receptors and modulate the fast synaptic transmission (fig. 1). NMDA receptors are widely distributed in the central nervous system. Several different subunits exist and functional NMDA receptors are heteromeric receptors. NMDA receptors conduct Na^+, K^+ as well as Ca^{2+} and opening and closing are very tightly regulated for instance by a voltage-dependent Mg^{2+} block. NMDA receptors are associated with synaptic plasticity and believed to be crucial for certain forms of synaptic memory. Non-NMDA receptors (AMPA and KA) are also widely distributed. Multiple subunits exist and the subunit composition is important for ion-conduction. For example AMPA receptors with a GluR2 subunit conduct Na^+ and K^+ but have a very low Ca^{2+} permeability, whereas receptors lacking GluR2 are 3-5 times more permeable for Ca^{2+}. For all types of glutamate receptors various splice variants and RNA editing further increases heterogeneity.[1]

Slow synaptic transmission is modulated by metabotropic glutamate receptors (mGluRs). Eight different receptors have been cloned and according to their sequence-homology, agonist pharmacology and the signal-transduction pathways to which they are coupled are clustered into three groups (fig.1). Different subtypes have different locations both on pre- and postsynaptic sites. Metabotropic glutamate receptors regulate both K^+ and Ca^{2+} channels.[1]

High concentrations of extracellular glutamate are neurotoxic and can cause signal spillover to neighboring synapses. Therefore it is essential for normal brain functioning that extracellular glutamate levels are kept low. Neurons and glia tightly control removal of glutamate from the synaptic cleft by the so-called glutamate-glutamine cycle.[2]

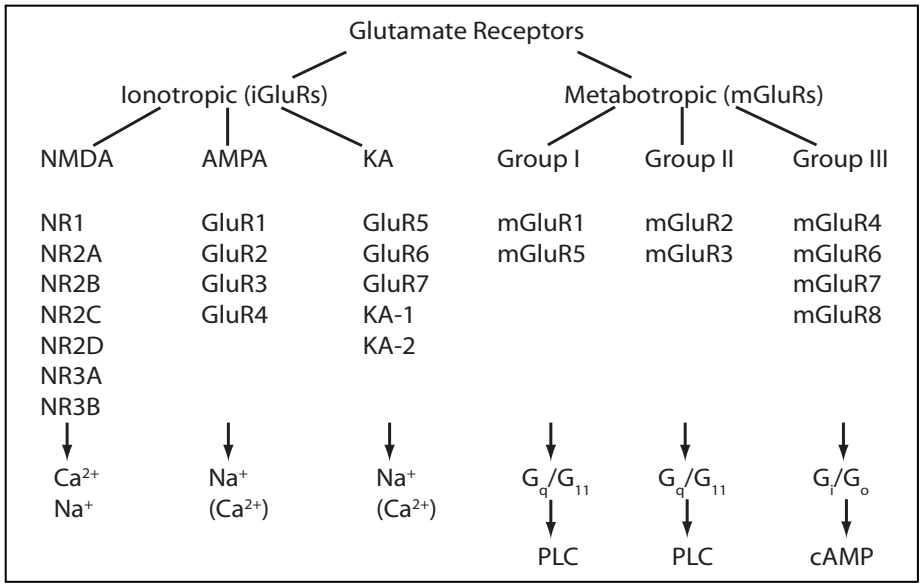

***Figure 1**. Ionotropic and metabotropic glutamate transporters.*

1.2 Glutamate-glutamine cycle

Extracellular glutamate is actively removed from the synaptic cleft by means of glutamate transporters, also called excitatory amino-acid transporters (EAATs). A large population of these transporters is present within or near the synaptic cleft. Both binding to and uptake by the transporters ensures a very rapid clearance process. 90% of the extracellular glutamate is taken up by glial glutamate transporters.[3] In glial cells, glutamate is converted into glutamine by the enzyme glutamine synthetase (GS, EC 6.3.1.2). Glutamine is a non-toxic amino acid and is transported back to the neurons. Phosphate activated glutaminase (PAG, EC 3.5.1.2) can metabolize glutamine into glutamate. This glutamate can be packed into synaptic vesicles by vesicular glutamate transporters (VGLUTs), thereby closing the glutamate-glutamine cycle (fig. 2).[2] In astrocytes approximately one-third of the glutamate is replenished by *de novo* synthesis of glutamate from glucose via the tricarboxylic acid cycle (TCA), which takes also place in neurons.[4]

To date five different EAAT subtypes have been cloned and characterized electrophysiologically and pharmacologically: EAAT1 (a.k.a. GLAST)[5,6], EAAT2 (a.k.a. of GLT1)[5,7], EAAT3 (a.k.a. EAAC1)[5,8], EAAT4[9] and EAAT5.[10] EAAT1 and EAAT2 are predominantly glial and the other three are neuronal. EAAT1, 2 and 3 can be found throughout the CNS, although EAAT1 is highly expressed in Bergmann glia of the cerebellum. EAAT4 is located mainly in Purkinje cells of the cerebellum and EAAT5 is only present in the retina.

Several EAAT subtypes are also expressed in peripheral tissues like liver, kidney and placenta. The amino-acid homology is approximately 50% between subtypes and 90% between species homologues (for review on EAATs see[11]). Recently, the crystal structure from an eukaryotic glutamate transporter homologue from *Pyrococcus horikoshii* (aerobe thermophillic archaebacterium) was resolved, revealing that the transporter acts as a trimer, sticking in the membrane as a bowl, with a concave aqueous basin facing the extracellular milieu.[12] These results correlate with a study

determining relative position of extracellular domains of human EAAT3,[13] also showing that the transporter is a trimer with a large extracellular vestibule. EAAT subunits work independently and small molecular motions are associated with glutamate uptake.[12,13]

The transport of glutamate against the concentration gradient is coupled to the Na^+ gradient. Biochemical studies suggest the cotransport of 2 or 3 Na^+, one H^+ and one negatively charged glutamate ion simultaneously with the counter-transport of 2 K^+ ions. Thermodynamically uncoupled anion transport is also seen in EAATs. The rate is dependent on the subtype, EAAT4 and 5 are highly permeable for anions like Cl^-, and this Cl^- transport is almost non-existent in EAAT1.[11]

Glutamine synthetase plays an important role in nitrogen metabolism in virtually all organisms from bacteria to plants and animals. In human, it is expressed in a wide variety of cell types and tissues. The enzyme catalyzes the conversion of glutamate and ammonium to glutamine driven by the hydrolysis of ATP. In the human brain, as stated above, it plays an important role in termination of the neurotransmitter signal of glutamate, but also in ammonium assimilation and detoxification.[14]

The mitochondrial enzyme phosphate activated glutaminase (PAG) is important in e.g. the hepatic urea synthesis and the renal ammoniagenesis but also in metabolizing the precursor glutamine into glutamate in neurons. Two isoforms are present: L-type and K-type, and both are localized in brain tissue, as well as a subtype specific localization in other tissues.[15] Cellular localization is contradictory,[16] some find expression of PAG in astrocytes, whereas others found PAG protein exclusively in neurons.[17]

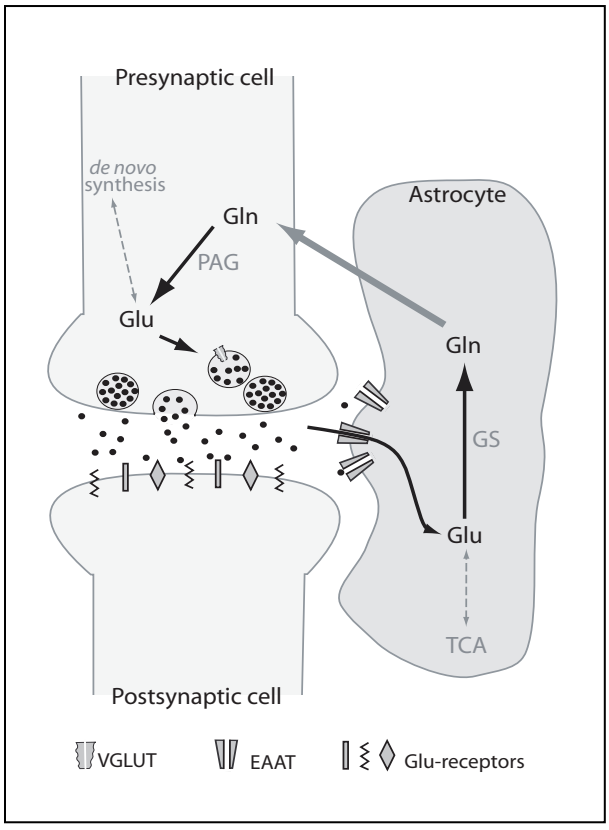

***Figure 2**. Glutamatergic neuro-transmission. Glutamate (Glu) released into the synaptic cleft activates Glu receptors located at the postsynaptic density, as well as (auto)regulatory Glu receptors at the presynaptic cell. The excitatory signal is terminated by removal of Glu from the synatic cleft predominantly by Glu transporters on astrocytes and is converted to glutamine (Gln) by the enzyme glutamine synthetase (GS). Alternatively, Glu can be metabolized by the tricarboxylic acid cycle (TCA). Gln is transported back to neurons, where is reconverted to Glu by phoshate activated glutaminase (PAG). Glu can also be formed in neurons by de novo synthesis through the TCA-cycle. Glu is stored in the presynaptic vesicles again by the vesicular Glu transporters (VGLUT).*

Glutamate is stored into synaptic vesicles by the vesicular glutamate transporter (VGLUT). Up to now, three subtypes have been identified named VLGUT1,2 and 3.[18-20] VGLUTs transport glutamate with a low affinity (~1mM), and transport is dependent on the vesicular proton electrochemical gradient generated by the vesicular proton ATPase. VGLUT1 and VLGUT2 have a largely complementary expression in all glutamatergic synapses throughout the brain. VGLUT1 is present in the hippocampus and cerebral and cerebellar cortex, while VGLUT2 is primarily localized in the diencephalon and rhombencephalon. VGLUT3 is expressed in neuronal populations throughout the CNS releasing other neurotransmitters like acetylcholine, GABA and serotonin. VLGUT3 is also located on peripheral tissues like liver and kidney.[21]

1.3 Excitotoxicity

It is long known that abnormal activation of glutamate receptors can lead to neuronal death, in particular of the postsynaptic cell. Olney called it *excitotoxicity* and gave it the following definition: "a phenomenon whereby the excitatory action of glutamate and related excitatory amino acids becomes transformed into a neuropathological process that can rapidly kill CNS neurons".[22] Excessive presynaptic glutamate release and/or impaired glutamate uptake can lead to excessive activation of postsynaptic glutamate receptors. Other factors can also contribute to the toxicity of glutamate; subtype and molecular properties of the postsynaptic glutamate receptors, the capability of the postsynaptic cell to restore the membrane potential, and the activity of downstream events after glutamate receptor activation. Excitotoxicity is a now a well established phenomenon that leads to neurodegeneration in a number of neurological disorders, including stroke, hypoxic-ischemic brain damage, epilepsy/status epilepticus and amyotrophic lateral sclerosis (ALS).

The mechanisms which lead to cell death can either be direct (acute) or indirect (secondary; fig. 3). Direct excitotoxicity involves a sequence of events. First, an influx of sodium and chloride ions, along with water, causes acute neuronal swelling that is reversible on removal of the agonist. Then there is influx of Ca^{2+} into the neuron through the ion channel of NMDA receptors, through AMPA receptors lacking the GluR2 subunit, or indirectly through the voltage-gated Ca^{2+} channels. In

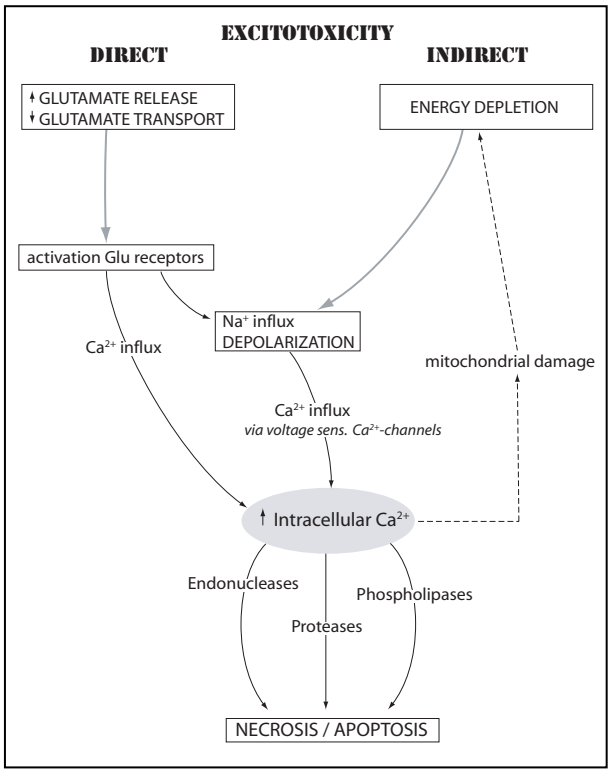

Figure 3. *Mechanism of direct and indirect excitotoxicity. See § 1.3 for more details.*

normal circumstances, the intracellular Ca^{2+} concentration is tightly regulated and below 100 nM. However, excessive activation of the glutamate receptors can destabilize this regulation and increase the intracellular Ca^{2+} concentration. This activates multiple Ca^{2+}-dependent enzyme cascades; such as protein kinase C, proteases, phospholipases, endonucleases, xanthine oxidase and nitric oxide synthase. Activation of these enzymes initiates necrotic or apoptotic cell death and can lead to the formation of free radicals. In addition, to decrease the level of intracellular Ca^{2+}, cells will activate energy dependent Ca^{2+}-transporters in the endoplasmatic reticulum, plasma membrane and mitochondria. In mitochondria, increased Ca^{2+} influx can lead to a loss of membrane potential, which in turn can lead to a decrease in ATP production and an increase in reactive oxygen species production.
Indirect excitotoxicity is a result of a compromised energy status of the neuron. This leads to a loss of the normal resting membrane potential, which causes an opening of the voltage-dependent magnesium block of the NMDA receptor. As a result, activation of glutamate receptors elicits a greater than normal response and further failure of energy-dependent ion pumps. (Reviewed in [23-25].)
As explained above, glutamate overexcitation leads to cell death. The mechanism of glutamate excitotoxicity is associated with several neurodegenerative diseases. I will describe in more detail two neurodegenerative diseases, amyotrophic lateral sclerosis and epilepsy, which pathogenesis is associated with glutamate excitotoxicity.

2 ALS

2.1 Clinical aspects

ALS is an adult-onset progressive neurodegenerative disorder characterized by loss of motor neurons. This results in progressive muscle weakness and wasting. Half of the patients die within 3 years, usually from respiratory failure. The incidence of ALS is 1-3 per 100,000 persons and men are 1.5 times more affected than women, although this tends to normalize after the menopause. The age of onset is usually between 50 and 60 years of age. The site of the disease onset can be either in the limb-area, resulting in weakness in arms and legs, or less frequent in the bulbar area, resulting in difficulties in swallowing and speech. Motor neurons controlling pelvic floor muscle and oculomotor neurons are relatively spared.[26] To date there is no effective treatment for this disease. Riluzole (Rilutek), the only FDA approved drug interferes with excitatory neurotransmission in the CNS and it has a modest effect on survival in patients with ALS.[27,28]
Neuropathological findings are degeneration of motor neurons in motor cortex, brain stem and spinal cord, accompanied by reactive gliosis, intracytoplasmic neurofilament abnormalities and axonal spheroids. In end-stage disease there is significant loss of large myelinated fibers in the corticospinal tracts and ventral roots as well as evidence of Wallerian degeneration and atrophy of the myelinated fibers. Also frontal and temporal lobe atrophy can be demonstrated.[26]

Approximately 5-10% of ALS cases are inherited (familial), but the majority of cases have no genetic component (sporadic). Sporadic and autosomal dominant familial ALS are clinically and pathologically similar. In 20% of the familial ALS cases, the disease is caused by dominantly inherited mutations in the gene coding for the Cu/Zn superoxide dismutase (SOD1).[29] Up to now more than 100 mutations throughout the entire gene have been found.[30] Sporadic ALS is most likely a multifactorial disease

2.2 Animal model

Transgenic expression of different human SOD1 mutants in mice[31-33] and rat[34] is used to study ALS. The commonly used mutation is a glycine substitution at position 93 (G93A). Those animals develop a paralytic syndrome that closely resembles the clinical and pathological hallmarks of ALS. Behaviorally these mice first develop fine tremor and thereafter weakness and atrophy of the proximal muscles that develop progressively. At the end stage, transgenic SOD1 mice are severely paralyzed and unable to eat and drink.[35]

The initial neuropathological finding is vacuolar degeneration of motor neurons. Dramatic motor neuron loss, an abundance of dystrophic neurites, a marked gliosis, some Lewy-body-like inclusions and motor neurons filled with phosphorylated neurofilament are seen in the end-stage, when the animals are severely paralyzed.[30,36] Although there is a high similarity between the neuropathology of these animal models and human ALS, there are also differences (see e.g.[30]). Nevertheless, these transgenic mice are most frequently used as a model of ALS.

2.3 Pathogenesis of ALS

ALS is a multi-factorial disease and several factors contributing to the pathogenic mechanism are proposed; 1) oxidative stress, 2) cytoplasmic protein aggregates (ubiquitin containing), 3) glutamate-mediated excitotoxicity, 4) disorganization of intermediate filaments, 5) disrupted retrograde axonal transport, 6) activation and proliferation of microglia, 7) mitochondrial dysfunction and 8) deficiency of neurotrophins (reviewed in[26]), which are all likely to converge to produce motor neuron degeneration (fig. 4a).[26] I will focus on the evidence indicating that excitotoxicity contributes to motor neuron degeneration in ALS.

The first indication that glutamate-mediated excitotoxicity contributes to the pathogenesis of ALS is the finding of increased levels of glutamate in the cerebrospinal fluid of ALS patients.[37,38] Glutamate toxicity is also shown in *in vitro* organotypic slice cultures, where blocking of glutamate transport increased the glutamate concentration in the culture medium and slow motor neuron degeneration was seen.[39] This leads also to the idea that diminished glutamate transport could be responsible for the elevated extracellular glutamate levels *in vivo*. A major loss of synaptosomal glutamate uptake was indeed shown in *post-mortem* brain tissue (spinal cord, motor cortex[40], fig. 4b), and this was accompanied by a marked decrease in EAAT2 protein expression.[41,42] Altered splicing of EAAT2 is thought to play an important role in the pathology of ALS.[43,44] The precise role of aberrant forms of EAAT2 in ALS needs to be further investigated, as mutations causing abnormal EAAT2 properties[45,46] and point mutations[47] are relatively infrequent and some splice

variants are widely distributed in normal and diseased brain.[48-50] Contradictory results are found in the mutant SOD1 model. Although decreased glutamate transport[51] and decreased expression of EAAT2 protein in the spinal cord[52] were shown in the SOD1 mutant mouse model for ALS, this was only present at the end-stage and not in early phases of the disease.[53,54] As human *post-mortem* tissue is also in an end-stage of the disease, it is possible that in human ALS the loss of glutamate transport and EAAT2 is also a consequence of disease progression rather than a cause of the disease. Another explanation for the difference between the human and SOD1 mutant mice model in neuropathology regarding the loss of EAAT2, is the presence of different subpopulations of patients. This is reflected in the fact that only in a subset of patients elevated cerebrospinal fluid levels of glutamate have been found.[38,55] SOD1 mice may not represent the neuropathology of all different types of ALS patients.

The high susceptibility of motor neurons in ALS can be explained partly by excitatory mechanisms. Motor neurons in the dorsal horn of the spinal cord express relative low levels of the GluR2 subunit of the AMPA receptor,[56-58] which makes the receptors on motor neurons more permeable for Ca^{2+}.[59] However, GluR2 is not completely absent on motor neurons and expression is comparable between normal and ALS subjects.[60] Posttranscriptional editing of the GluR2 mRNA at the Q/R site results in an AMPA

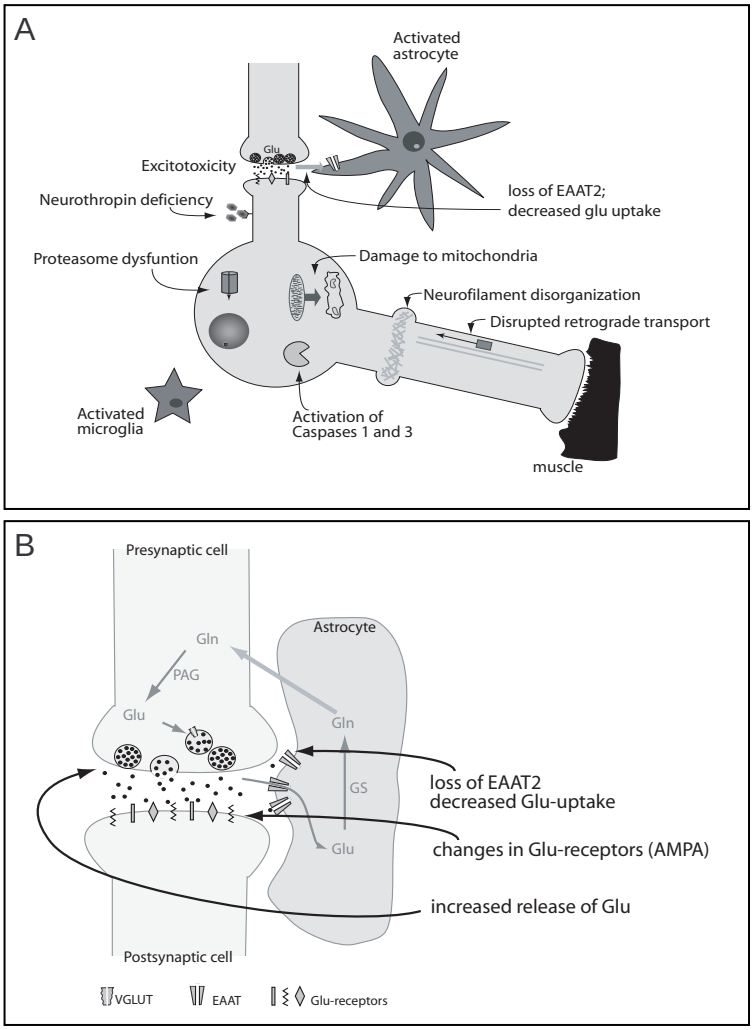

Figure 4. *A: Convergence of several pathogenic mechanisms in ALS. B: Excitotoxic hypothesis of ALS in more detail.*

receptor containing the edited GluR2 (GluR2R) subunit. These receptors are less permeable for Ca^{2+} than the unedited form (GluR2Q). In motor neurons from ALS patients, it is shown that the editing efficiency in specific regions is decreased dramatically as compared to motor neurons from healthy controls.[60,61] Another form of posttranscriptional editing (flip/flop) regulates the formation of slow and fast desensitizing AMPA receptors. A decrease in flop variants is shown in the ventral segment of spinal cord tissue from patients with ALS, leading to a higher percentage of slow desensitizing AMPA receptors.[62] Finally, motor neurons have relative low Ca^{2+} buffering capacity,[63] which renders them even more vulnerable to a glutamate elevation and/or changes in AMPA receptors.

3 Epilepsy

3.1 Clinical aspects

Epilepsy is a chronic brain disorder characterized by recurrent seizures, which are caused by disordered, synchronous, rhythmic firing of populations of neurons. The prevalence is 0.4%-0.8% and the incidence of epilepsy is about 0.5%.[64] Clinical manifestations of seizures range from a major motor convulsion to a brief period of lack of awareness. The clinical features of seizures are determined by the normal functions localized in the region of cortex in which neurons fire, the time course and discharge propagation. Epilepsy is a not a specific disease or even a single symptom, but rather a broad category of symptoms caused by disordered brain excitation, that in itself may be secondary to a variety of pathologic processes, like head trauma, neoplasm's, congenital disorders or infections.

Epilepsy is treated with a variety of drugs. Drug therapy, despite the name antiepileptic-drugs (AED), is directed at control of symptoms i.e. the suppression of seizures by chronic administration of these drugs. Overall in approximately 30% of patients seizures are not well controlled. None of the conventional and new generations of AEDs appear to be anti-epileptogenic.

Epileptic seizures are divided in partial and general seizures. Partial seizures have in general clinical and electroencephalographic evidence indicating the initial participation of a part of one hemisphere. When consciousness is not impaired, the seizure is said to be simple, when consciousness is lost, the seizure is classified as complex. In partial seizures, motor manifestations are predominantly unilateral. Partial seizures can evolve into generalized seizures (secondary generalization) with bilateral tonic-clonic convulsions. In generalized seizures, the first clinical signs indicate the involvement of both hemispheres. The motor convulsions are bilateral. Generalized seizures can be nonconvulsive (absences) or convulsive (e.g. myoclonic, tonic-clonic or atonic seizures). A status epilepticus (SE) occurs when a seizures persist for a sufficient length of time or is repeated frequently enough that recovery between attacks does not occur.[65]

Epilepsies and epilepsy syndromes are classified in four main classes; localization related epilepsies, generalized epilepsies, symptomatic epilepsies and special syndromes. Localized and generalized epilepsies are further subdivided according

their cause; idiopathic, symptomatic or cryptogenic. Approximately 50% of epilepsy patients suffer from localization related epilepsies. In approximately 80% of these patients, the seizures originate in the temporal lobe. Temporal lobe epilepsy (TLE) patients therefore form a large patient group and pathogenesis of TLE is studied quite extensively both in humans and animal models. Therefore I will focus on TLE in further discussion.

3.2 Temporal lobe epilepsy

Patients with TLE have seizures which usually involves symptoms like: sensation of epigastric rising, emotional changes (most commonly fear) and occasionally olfactory or gustatory hallucinations. The seizures may become complex and patients then have an impairment of consciousness: typically a stare with arrest of motion followed by altered responsiveness, oroalimentary and gestural automatisms and later reactive automatisms lasting typically one to two minutes. Postictal amnesia and disorientation may occur.[66]

TLE is divided in three broad categories: 1) TLE associated with hippocampal sclerosis referred to as HS-TLE or mesial temporal lobe epilepsy (MTLE), 2) TLE associated with specific lesions, such as vascular malformations, tumors, and dysplasias, referred to as “lesional partial epilepsy” or non-HS-TLE and 3) TLE with an unknown etiology.[66]

About 20% of patients with TLE develop drug refractoriness.[67] If drug treatment fails, an alternative option is the surgical removal of the epileptogenic zone, i.e. a part of the temporal lobe and the hippocampus. A prerequisite is that the epileptic focus is single, clearly diagnosed and not lying in eloquent areas. Surgical treatment has a favorable prognosis, 50-60% of the patients become seizure-free after temporal lobe resections at 2 years after surgery.[68] Epilepsy duration seems to be the most important predicting factor for seizure freedom at long term after surgery for HS-TLE.[69] The resected tissue is used for neuropathological diagnosis and for research purposes.

Patients with HS-TLE usually start with seizures at an age between 5-10 years and most often respond initially to AED treatment. During adolescence or later the seizures recur and often become intractable. Hippocampal sclerosis (HS) is a scar lesion in the hippocampus and is characterized by neuronal degeneration and by astrogliosis. Preoperative T2-weighted MRI images show increased hippocampal signal intensity and also further abnormalities in the temporal lobe e.g. a decreased gray-white matter demarcation in the temporal lobe.[70] Three histological features are seen in HS: 1) neuronal cell loss and consequent astrogliosis, 2) granule cell dispersion and 3) mossy fiber sprouting. (For detailed description of the anatomy of human hippocampus see box 1)

Neuronal cell loss in HS is not uniform in the hippocampal formation. Highly vulnerable areas are the CA1, CA3 and the hilus/CA4, whereas the CA2 pyramidal cells and the granule cells are relatively resistant. Also some pyramidal neurons in the subiculum and the presubiculum disappear.[71]

Another feature of HS is granule cell dispersion.[72] The granule somata are dispersed

and form a wider than normal granule cell layer, the somata extend into the molecular layer to varying extent, creating an irregular boundary between the lamina. The extent of granule cell dispersion appears to be related to the amount of cell loss in the polymorphic layer of the dentate gyrus. The mechanism of granule cell dispersion is not clearly defined, a neuronal migration disorder has been hypothesized as the cause but alternatively, dispersion could be induced by structural plasticity induced by an initial precipitating injury early in life.[73]

The third feature characterizing HS is the reorganization of the mossy fibers, called mossy fiber sprouting. Mossy fibers that normally innervate hilar neurons, in particular mossy cells and CA3 pyramidal cells send collaterals to the inner third of the molecular layer of the dentate gyrus.[74,75] Mossy fiber sprouting is easy to identify using Timm's staining, a silver precipitating redox reaction with the zinc ions that are present in high concentrations in the synaptic boutons of mossy fibers.[76]

3.3 Animal models of epilepsy

In temporal lobe epilepsy, it is possible to use human resected hippocampal tissue to examine pathological changes. However, it is difficult to determine a causal relationship between of changes in expression pattern of certain proteins and/or the changes in cell number found in this tissue to the process of epileptogenesis. To study the mechanism of epileptogenesis, animal models of epilepsy are essential. Nowadays a large number of animal models of epilepsy are used which can be divided in genetic models and models of acquired epilepsy e.g. the kindling model and post-status epilepticus (SE) models (chemically- or electrically-induced).

In the post-SE models, the application of a convulsant chemical or electrical stimulation causes the initial insult and causes a self-sustained SE that lasts for several hours (acute phase). In the following period (latent phase), functional and structural reorganization occurs, but the animal shows no sign of clinical seizures symptoms yet. Dependent on age of the animals and model used; at some point animals enter the chronic phase, in which spontaneous seizures occur with increasing frequency. In the following paragraphs the most widely used models of epilepsy will be briefly described. Both rats and mice are used in all models, except of course the genetic models, which are either rat or mice strains.

3.3.1 Genetic models

Various mice and rat strains with an inborn susceptibility for epilepsy or inbred mice with an induced mutation are present. For example in the genetic absence epilepsy rat from Strassbourg (GEARS)[77] and the EL mice[78], seizures are inherited as an multifactorial trait and are considered as model for human complex partial seizures with secondary generalization. Other genetic models for epilepsy have single gene loci e.g. in Ca^{2+}-channels in the tottering, lethargic, stargazer and waggler mice strains. These mice have also other defects like ataxia (for review see [79]).

Secondly, although not specifically developed to model epilepsy, genetically modified mice, in which various genes are knocked out, seizures are often part of the phenotype.

3.3.2 Kindling model

The kindled model of partial epilepsy is perhaps the most studied model of epileptogenesis. It was introduced in 1967 by Goddart.[80] Kindling is the repeated subconvulsive high frequency electrical stimulation of limbic structures in the amygdala, hippocampus or enthorhinal cortex. After repeated stimulations the animal displays both behavioral and electrographic seizures that spread to become secondarily generalized. Usually in standard kindling procedures, the seizures are not spontaneous but evoked. No obvious loss of hippocampal pyramidal neurons is observed.[81] On the other hand, over-kindled rats, exhibit spontaneous seizures and the pathophysiology is very similar to that of human MTLE, e.g. structural and functional changes characterized by neuronal cell loss, gliosis, neurogenesis and mossy fiber sprouting are observed.[82] This model can be used to study epileptogenesis, and it is widely used to study AEDs, although a major drawback is that the drugs have to be delivered before the kindling stimulation, thereby preventing the expression of the kindled seizure rather than by affecting the processes underlying the hyperexcitability (for reviews see [83-85]).

3.3.3 Electrically-induced status epilepticus model

A self-sustained status epilepticus is elicited by sustained electrical stimulation of the hippocampus (via stimulation of the perforant path[86,87], the angular bundle[88] or the CA3 of the ventral hippocampus[89]), the lateral or basolateral nucleus of the amygdala[90] or other limbic brain regions. After a latent period, animals develop recurrent spontaneous seizures. These models are characterized by the fact that neuropathological changes reminiscent of MTLE and recurrent spontaneous seizures develop after the status. Drawback of these models is that they are technically more difficult and sometimes the rats are anesthetized. The neuropathological findings are often more widespread throughout the brain. An advantage of these models is the induction of the SE without the application of toxins (for reviews see [83,84]).

3.3.4 Chemically-induced status epilepticus model

In these models, high doses of toxic agents are applied locally or systemically. Typically, kainate[91] or pilocarpine[92] are used, but also other chemicals like ferric ions[68] are used. By these chemicals an SE is induced which can last for hours if untreated. After a latent period a high percentage of animals develop spontaneous recurrent seizures (SRS). Characteristic for these models is the high mortality rate. The neuronal damage resembles that of human MTLE, but is often more widespread and severe. In the following section the pilocarpine model is highlighted.

3.3.5 The pilocarpine model

Pilocarpine is a cholinergic agonist activating the muscarinic acetylcholine (ACh) receptors. The long-term effects of a single peripheral dose were first observed by Turski *et al*. in 1983[92] and include neuropathological damage; cell loss in the hippocampal subfield CA1 and CA3, in the hilus of the dentate gyrus, the septum, olfactory tubercle, amygdala, piriform cortex, neocortex and thalamic nuclei, and

mossy fiber sprouting is also seen. Spontaneous recurrent seizures are electrographically characterized with paroxysmal hippocampal discharges that rapidly spread to cortical regions and behaviorally by facial automatisms, head nodding, forelimb clonus, rearing and fall.[93] A number of AEDs have proven to be effective in treatments of SRS in this model, phenobarbital, carbamazepine, phenytoin and valproic acid, but ethosuximide was not effective.[94]

The human developing brain is differentially vulnerable for seizures and epileptogenesis. For example the NMDA subunit composition and activation kinetics is different in a way that enhances NMDA mediated excitation in immature neurons favoring hyperexcitability.[95] It is known that in human epilepsy the initial precipitating injury often occurs at an early age.[96] Therefore, animal models including the pilocarpine model, are thought to reflect the human situation more accurately when the epilepsy is induced at an early age. As in humans, the susceptibility to seizures in rats is also age-dependent.[97] The susceptibility peaks during the third week of live. At a younger age, lethality during the pilocarpine induced SE is very low, neuronal cell death in most areas is low or very low, mossy fiber sprouting is absent and a low percentage of animals develops SRS.[98]

One adaptation of the original model from the group of Turski and Cavalheiro has been made to reduce mortality due to the pilocarpine-induced SE. Pretreatment with lithium-chloride and subsequent 5-10 times lower dose of pilocarpine reduced the mortality and side effects but behavioral, electrographic, metabolic of histopathological findings are alike. Lithium is thought to influence cholinergic function by increasing release of ACh, changing the muscarinic binding or changing the inositol phospolipid second messenger system,[99] but is also inhibiting glutamate uptake.[100] Nowadays most pilocarpine studies use pretreatment with lithium.

3.4 Pathogenesis of epilepsy

A large number of different epilepsy syndromes exist. In the first place the generalized epilepsies and the focal epilepsies, but for instance also etiology, age related epilepsies etc. At least 30% of the epilepsies have a genetic component. Common forms of epilepsy are considered polygenic in combination with environmental factors. Several hypotheses have been proposed, but it is likely that, more than one mechanism exists.

Several factors can influence the hyperexcitability of tissue. The first and most obvious candidates are ion channels, involved in rest-potential of the membrane and/or action potentials. Secondly, changes in network connectivity may change network excitability (TLE–hippocampal sclerosis). Thirdly, probably underestimated, astrocytes and fourthly, proteins involved in the inhibitory and excitatory neurotransmission.

In idiopathic epilepsy, a small number of families have been identified with mutations in several ion channels. These include potassium, sodium, and chloride channels, nicotinic ACh receptors subunits and $GABA_A$ receptor subunits. Table 1 gives an overview of most of the genes coding for ion channels implicated in various forms of epilepsy (for review see [101]). The inheritance pattern in these families is largely monogenetic, but commonly with reduced penetrance, indicating modifications by

environmental factors or other (epi-)genetic influences. Only a limited number of mutations and families have been identified, although a large number of patients suffer from idiopathic epilepsy. This indicates that a large number of yet unidentified genetic factors may exist.[101]

Two hypotheses regarding TLE have been proposed indicating the importance of network connections. As stated above, mossy fiber sprouting is seen in MTLE hippocampi. Hilar mossy cells prove to be one of the most vulnerable cells in the hippocampus. Two possible mechanisms have been proposed to explain why the loss of these mossy cells results in granule cell hyperexcitability. First, the sprouting hypothesis[102] states that an initial precipitating injury causes mossy cell death. As a reaction to the loss of target neurons, granule cells sprout and form new synapses back on themselves. This excitatory feedback loop may contribute to seizure generation. The second hypothesis is the dormant basket cell hypothesis.[103] As mossy cells die, their tonic excitatory projection to basket cells is lost. The basket cells, which are inhibiting granule cells, become dormant and permanent disinhibition of the granule cells is the result. In the recent past, results from several studies have cast serious doubts on the necessity of mossy fiber sprouting and specific neuron cell loss in epileptogenesis.[104,105] Also the dormant basket cell hypothesis needs revision, because the altered GABAergic transmission seems not to be related to the dormancy of hilar basket cells.[106]

The third factor thought to contribute to epileptogenesis are astrocytes.[107] Astrocytes are a remarkable heterogeneous population of cells, they have been shown to posses their own form of excitability (the Ca^{2+}-wave), they can react to various neuroactive substance and express many types of ion channels and neurotransmitter receptors. One of the important tasks of glial cells is the maintenance of extracellular K^+ homeostasis. In several forms of epilepsy reactive gliosis takes place (e.g. in epilepsy related to traumatic head injury, TLE, tumor related epilepsy). Reactive astrocytes at the site of neurodegeneration, have changed K^+ currents and a decreased capability to control extracellular K^+.[108,109] This is shown both in the CA3 area of the hippocampus

Table 1: Ion channel mutations in idiopathic epilepsy

Voltage gated ion channels	Syndrome
KCNQ2, KCNQ3	Benign familial neonatal convulsions (BFNC)
SCN1B, SCN1A, SCN2A	Generalized epilepsy with febrile seizures plus (GEFS+)
SCN2A	Benign familial neonatal infantile convulsions (BFNIC)
SNC1A	Severe myoclonic epilepsy of infancy (SMEI)
CLCN2	Idiopathic generalized epilepsy (IGE)
Ligand gated ion channels	
CHRNA4, CHRNB2	Autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE)
GABRG2	Childhood absence epilepsy / febrile seizures (CAE/FS), GEFS+
GABRA1	Familial juvenile myoclonic epilepsy (FJME)

CHRNA/B= α/β subunit, nicotinic ACh receptor, CLCN=voltage gated chloride channel, GABRG/A=$GABA_A$ receptor, γ/α subunit, KCNQ=voltage gated K^+ channel, SCN= voltage gated Na^+ channel.

after a traumatic head injury (in vivo animal model[110,111]). and in human HS-TLE tissue in CA3 astrocytes[112-114] and in hilar astrocytes.[115] This chronic pathological state can be induced by prolonged inflammation and/or the lack of functional neurons in their neighborhood.[107]

The fourth factor contributing to epileptogenesis are pathological changes in inhibitory and excitatory neurotransmission. The literature on this subject is extensive, changes are found in GABAergic neurotransmission and glutamatergic transmission in both animal models and in human tissue. Here I will focus on changes found in the glutamate-glutamine cycle in human TLE-hippocampi (fig. 5). The reader is referred to an excellent, although somewhat older review that deals with glutamatergic changes by Meldrum *et al.*[116] and to a review regarding GABA changes by Sperk *et al.*[117]

The glutamate metabolism is changed during seizures. In the human hippocampus it is found that just before[118,119] and during a seizure the extracellular glutamate concentration is increased[118] and the rate of glutamate-glutamine cycling is decreased in the human epileptic hippocampus.[120] In rat, it is shown with microdialysis that after pilocarpine and kainate seizures glutamate concentrations increase both in adult and juvenile animals, regardless of the different nature of the seizure inducing chemicals.[121,122] Changes in glutamate and glutamine content are also found in amygdala kindled rat, in the ipsilateral hippocampus using *in vitro* ^{1}H NMR spectroscopy.[123]

Several studies describe alterations in glutamate transporter expression in human TLE. A moderate general decrease in EAAT1 immunoreactivity was reported in the hippocampus from TLE patients with hippocampal sclerosis (HS) compared to patients without sclerosis (non-HS) or to autopsy controls. Both mRNA and protein were decreased and most pronounced in the CA4, the PML and the SGL,[124] whereas another study found no changes in EAAT1.[125] More dramatic changes were found in EAAT2. Both studies reported a large decrease in EAAT2 in areas of neuronal cell

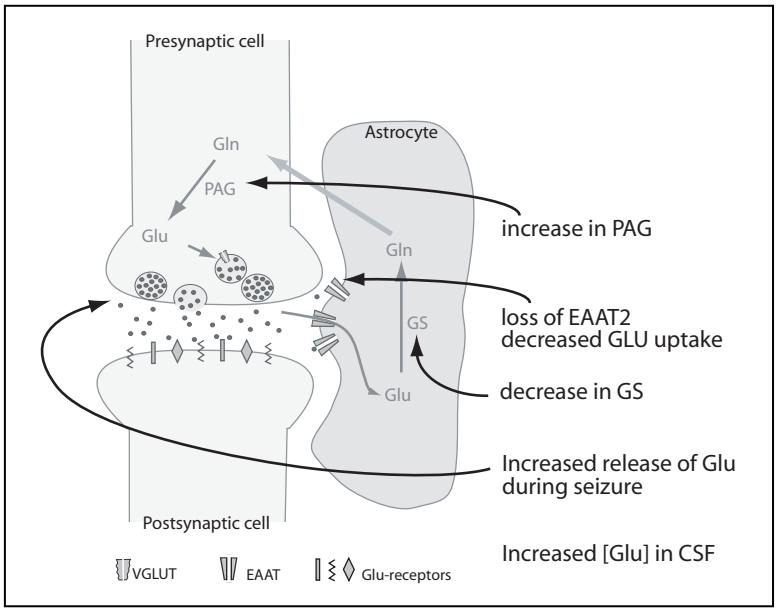

Figure 5. *Excitotoxicity in human TLE*

loss in hippocampi from TLE patients with HS compared to the non-HS group.[124,125] In the same patient group an increase in EAAT3 in the remaining neurons was found.[124,125] TLE patients with HS show a large reduction in the enzyme glutamine synthetase.[126,127] The glutamate synthesizing enzyme, phosphate activated glutaminase (PAG), which is confined to the mitochondrial department in neurons, is specifically increased in the subiculum of MTLE patients compared to non-MTLE patients (Hammer *et al.* poster abstract FENS Forum 2004). These findings are summarized in figure 5.

Box: Hippocampal Anatomy

The human hippocampus is situated in the floor of the temporal horn of the lateral ventricle. The rat hippocampus is a large C-shaped structure underneath the posterior and temporal neocortex. Although the brain location and orientation in human and rat differ, the basic structure is largely identical (for reviews see [128-130]). The hippocampal formation includes the hippocampus proper (cornu Ammonis), the dentate gyrus and the subiculum and is originating from relatively simple cortex. The three dimensional structure of the hippocampus is rather complex as it consist of two major U-shaped interlocking sectors, one the dentate gyrus, the other the cornu Ammonis. In the rat these layers are more or less identical along the septo-temporal axis, but in the human hippocampus they are more intertwined in the proximal (head) and distal (tail) parts of the hippocampus.

The Cornu Ammonis

The human hippocampus proper is divided by Lorente de Nó[131] in four subfield; CA1, CA2, CA3 and CA4 (fig. 6). The primary neurons in the CA fields are the pyramidal neurons. The CA1 field is a large region of small, scattered triangular pyramidal neurons adjacent to the subiculum. In rodent the pyramidal cells are more densely packed. The CA2 is larger and more pronounced in humans than in rodents and consist of large ovoid densely packed pyramidal neurons adjacent to CA1. CA3 is located in the curve of the hippocampus and the pyramidal neurons are large, ovoid as in CA2, but less densely packed. The region between the legs of the dentate gyrus is called the CA4 or dentate hilus and contains few, scattered, ovoid and large neurons.
Although it is basically a three-layered structure, the cornu ammonis is generally divided into several layers (fig. 6). From the outside (ventricular surface) to the inside (hippocampal fissure) there are: (1) the *alveus*, containing the axons of the hippocampal and subicular neurons, directed towards the fimbria or the subiculum; (2) the *stratum oriens*, a layer between the alveus and the pyramidal cell bodies, containing the basal dendrites of the pyramidal cell and some of the basket type interneurons as well as the passing pyramidal axons; (3) the *stratum pyramidale*, or pyramidal layer, which is dominated by the cell bodies of the pyramidal neurons; (4) *stratum radiatum*, containing the proximal segments of the apical dentritic tree of the pyramidal cells, the parallel orientation of the dendrites gives this layer a striated appearance; (5) *stratum lacunosum* and (6) *the stratum moleculare*, consisting of the distal segments of the apical dendritic tree of the pyramidal cells. These layers are

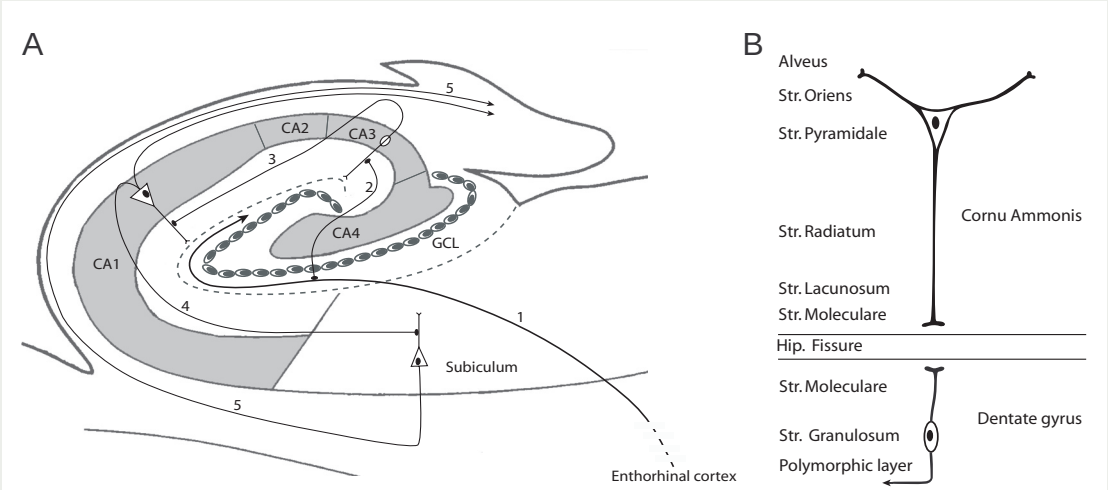

Figure 6. *Human hippocampus anatomy.* ***A****: Schematic drawing of the different sublayers of the hippocampus and schematic representation of the trisynaptic pathway. 1=perforant path, 2=mossy fibers, 3=Schaffer collateral, 4= major output of CA1 to subiculum, 5= efferent fibers to alveus and fimbria.* ***B****: The different laminae of the cornu Ammonis and the dentate gyrus.*

sometimes considered as one layer. The stratum lacunosum also contains numerous axons, formed by the perforant fibers and the Schaffer collaterals oriented perpendicular to the pyramidal dendrites in the stratum radiatum. In the CA3 field an additional layer is recognized: the *stratum lucidum* interposed between the pyramidal cell bodies and the stratum radiatum, containing the mossy fibers originating from the dentate granule cells.

The Dentate Gyrus

The dentate gyrus (or fascia dentata) is also a U-shaped structure, the blade inside and adjacent to the hippocampal fissure is called the buried or infra-pyramidal blade, the opposite blade is referred to as the exposed of supra-pyramidal blade and the connecting portion is called the crest. The dentate gyrus has three layers. (1) The *stratum granulosum* containing the small, round and densely packed cell bodies of the granule cells (primary cells of the dentate gyrus). (2) The *stratum moleculare* is a thick layer containing the dendritic tree of the granule cells; its external two-third receives fibers from the perforant pathway and the inner one-third is occupied with commissural and septal fibers. (3) The *polymorphic layer* in the hilus which merges with the CA4 field and contains the granule cell axons that gather together to form the mossy fiber bundle and various interneurons e.g. excitatory mossy cells and inhibitory basket cells.

Hippocampal circuitry

Afferent pathways

The input to the hippocampus consists of cortical and subcortical sources from the contralateral hippocampus, associational fibers from the ipsilateral hippocampal regions, dentate gyrus, subiculum and entorhinal cortex. The fibers generally release

excitatory amino acids and some may co-release various peptides. Further on, these projections originate from the dorsolateral prefrontal cortex, orbitofrontal, temporal and parahippocampal cortices, olfactory bulb, amygdala, medial septum, raphe nucleus locus coeruleus and the thalamus. Subcortical input sources from the hippocampus include the septum (ACh, GABA), hypothalamus, raphe nucleus (5HT) and locus coeruleus (NA).

Trisynaptic pathway

The major intrinsic pathway in the hippocampus passes three excitatory, glutamatergic synapses and is therefore called the trisynaptic pathway (fig. 6). This pathway is unidirectional. The dentate gyrus receives major input from the entorhinal cortex via the so-called perforant pathway that crosses the hippocampal fissure. The granule cells of the dentate gyrus project via their mossy fibers to the CA3 field of the hippocampus. Pyramidal cells of the CA3 field give rise to collateralized axons that terminate within CA3 as associational connections and also provide the major input to the CA1 field of the hippocampus, the so-called Schaffer collaterals. The CA1 field of the hippocampus projects to the subiculum (see efferent pathways). In the early days, the trisynaptic pathway was thought to be organized in a strictly lamellar fashion. More sophisticated anatomical studies provided evidence that the mossy fibers are indeed organized in a lamellar fashion, but the other projections also project extensively along the septotemporal axis of the hippocampus.[132]

Local circuitry

Within subregions of the hippocampus and dentate gyrus, interneurons and principal cells form local synaptic circuits both directly exciting (neighboring) cells and indirectly inhibiting (neighboring) cells. For example, granule cells send extensive collaterals to many interneurons in the hilus, particularly mossy cells, which in turn contact other hilar interneurons and granule cells. CA3 pyramidal cells do not only have Schaffer collaterals, but also extensive axon collaterals that synapse on neighboring CA3 pyramidal cells and inhibitory neurons. Within the CA1, pyramidal neurons send excitatory collaterals to several classes of interneurons.

Efferent pathways

The major output projections from the hippocampus are the axons of the CA1 pyramidal neurons. They leave the hippocampus via the alveus and project to the subiculum, entorhinal cortex, lateral septal nucleus, olfactory bulb, nucleus accumbens, amygdala and the hypothalamus. Via the subiculum and the entorhinal cortex, indirect projections go to numerous temporal, sensory and associational neocortical areas as well as to the amygdala, thalamus and mammillary bodies. The CA3 area (pyramidal and non-pyramidal collaterals) projects to the lateral septal nucleus.

4 Blood markers for neurological diseases

4.1 Introduction

In many neurodegenerative diseases the symptoms occur after a long delay. The pathogenesis in the central nervous system may have started many years before. Also, diagnosis of those diseases is largely based on clinical symptoms, since it is not possible to examine the brain tissue during life.

Temporal lobe is an exception in so far that brain tissue can be examined during the course of the disease. As discussed above, the focus of the epilepsy can be resected as treatment for TLE and this brain tissue is available for research purposes. Even this tissue has major drawback, as it is thought to be in an end-stage of the disease and reflects only a subgroup of TLE patients because these patients are drug refractory, suffer for years from seizures and have been treated with several anti-epileptic drugs before resection takes place. Post-mortem tissue is used as control tissue with obvious drawbacks.

Both for the understanding of the pathogenic mechanism and for treatment of the patient it is important that the neurological illness is diagnosed as early as possible. Therefore, new diagnostic and *in vivo* examination techniques are important. For example general diagnostic techniques, like imaging, are being developed and improved, e.g. high resolution magnetic resonance imaging (MRI), functional MRI (fMRI) and positron emission tomography (PET) are used to examine the brain tissue in a non-invasive way and give information about structure, scarring, water content and, in the case of PET, information about general metabolism (glucose and oxygen). Nuclear magnetic resonance (NMR) is used to examine biochemical processes (e.g. the glutamate-glutamine cycle[120,133]) Also more disease specific techniques like magnetoencephalograms (MEG)[134], which localize the source/focus of the epilepsy at a higher resolution than standard EEG, are being developed.

An alternative approach to find new tools that can serve as early diagnostic markers, but also may predict responsiveness to drugs, is the use of peripheral tissue or body fluids. Although cerebrospinal fluid is an obvious candidate, it is difficult and painful to obtain. On the other hand, blood has none of these drawbacks. It is easy and without risks to obtain. Therefore I will focus on markers of neurological disease in blood and blood cells.

4.2 Blood and blood cells

Human blood is composed of plasma (55%) and formed elements (cells and cell fragments, 45%). Plasma consists mostly of water and proteins like albumins and globulins. A small part of the plasma consists of dissolved substances, like ions, nutrients, waste products, gases and regulatory substances.

All blood cells originate from one pluripotent stem cell in the bone marrow (fig. 7), which descendants become differentiated along particular lineages. The three cell types present in blood are erythrocytes (biconcave disks without nucleus), leukocytes (white blood cells, various types) and thrombocytes (also called platelets, small cell

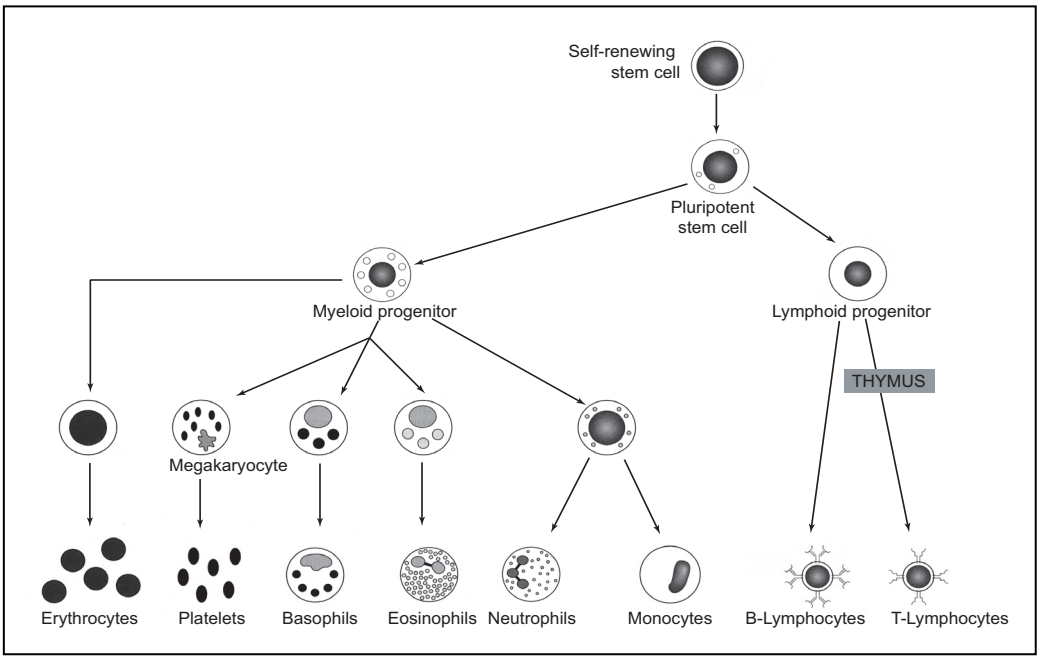

Figure 7. *Different blood cells and their progenitors.*

fragments). Erythrocytes main function is transporting oxygen and carbon dioxide throughout the body. Blood platelets are very important in blood coagulation. Leukocytes are the cellular components of the immune system. There are different subtypes, lymphocytes (T- and B-lymphocytes), granulocytes (neutrophils, basophils, eosinophils) and monocytes (precursor of macrophages) which all have a different and specific function in the immune system (see for example[135]). The normal distribution of different cell types is listed in figure 8.

The central nervous system is also dependent on blood for e.g. its oxygen and glucose supply. The brain is carefully shielded for abnormal variations in its environment as well as from potentially toxic molecules. The blood-brain-barrier (BBB) is important for protection and homeostasis of the brain. The BBB is the result of tight junctions between neighboring capillary endothelial cells. As a result, substances that traverse the wall of brain capillaries have to move through the endothelial cell membranes and are accordingly dependent on the molecular solubility in lipids. Nevertheless, many ions and molecules (e.g. glucose) that are not readily soluble in lipids move through the BBB by means of specific transporters.[136] For the treatment of neurological diseases it is essential to develop drugs that permeate the BBB. But also the disease itself can affect the BBB. For example during and shortly after seizures the selective permeability of the BBB is temporarily lost (reviewed in [136]). This leads to disease related cross-talk between brain and blood.

4.3 Blood markers for neurological diseases

Early literature on blood markers for neurodegenerative diseases is quite extensive. Although some differences were found, up to now none of these are used in clinical practice. This is partly due to the lack of knowledge on the pathogenesis and available experimental techniques. Attempts have been made in various disorders, e.g. migraine, depression, bipolar disorder, schizophrenia, Alzheimer's disease (AD),

Parkinson's disease, epilepsy and ALS. The attempts to find blood markers can roughly be divided in two main strategies, one that is directed to find a marker of the disease itself and can be used in diagnosis or treatment decisions and the second is directed to find signs that are a reflection of medication. Both of them are directed at proteins or molecules that are in one way or the other linked with the pathogenesis or therapeutic targets. Here, I will focus on amyloid precursor protein changes in AD, as an example of changes found in blood that correlate with changes found in brain and on changes found in blood plasma and cells of epilepsy and ALS patients.

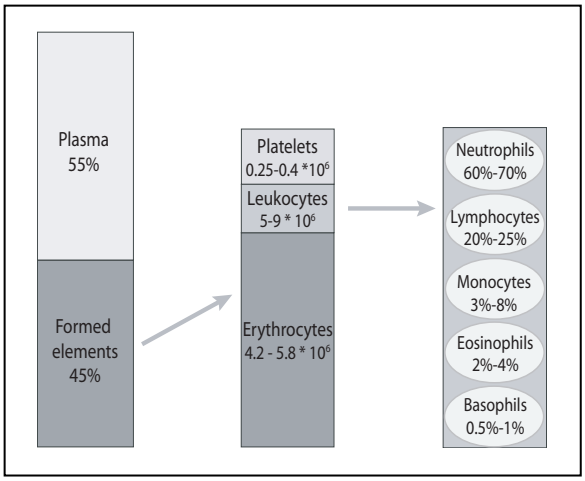

Figure 8. *Normal distribution of blood cells. Approximate values in a normal adult.*

4.3.3 Alzheimer's disease

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by a progressive cognitive and memory decline, increasingly frequent with advancing age. Neuropathological findings are selective neuronal cell death associated with two hallmark pathological lesions, extracellular amyloid deposits in the form of senile plaques and intracellular, neurofibrillary tangles (NFTs). Since the finding of β-amyloid peptide in senile plaques from AD patients, interest is gained in this protein in peripheral tissues as well. Amyloid β-peptide (Aβ) originates by proteolytic processing of β- and γ-secretases from amyloid β precursor protein (APP). Due to an decreased α-secretase activity, increased or changed β- and γ-secretase activities and maybe a decreased breakdown system are thought to be responsible for an increased amount of Aβ peptide so that plaques can be formed.[137] APP exists in various isoforms due to variable splicing of its mRNA. It is thought that peripheral tissues can either mirror pathologic changes in the brain or even contribute to Aβ deposition.[138] Platelets contain three isoforms of APP. The ratio between high kDa (130) and low kDa (106-110) isoforms is decreased in platelets of AD patients and this decline is correlated with the severity of the disesase,[138-140] although normal mRNA levels of APP were found in those platelets.[138] After a two year follow up of patients with mild cognitive impairment, a decline in this ratio was found in patients that progressed into Alzheimer type of dementia but was not changed in stable or non-Alzheimer dementia patients,[141] suggesting that the ratio might have predictive value. The APP isoform ratio in platelets was increased towards normal levels after 30 days of treatment with an cholinesterase inhibitor (donepezil) which exerts some cognitive ameliorating efficacy.[142] All enzymes for normal proteolytic cleavage of APP are present in platelets.[143] ADAM10 (an α-secretase) and its product αAPP is reduced in platelets from AD patients[144] as well as in CSF. APP metabolism changed toward normal after treatment with an cholinesterase inhibitor; ADAM10 protein and product was

increased, and β-secretase product was decreased.[145] Serum cholesterol had an effect on the APP isoform ratio, since AD patient with high serum levels of cholesterol have lower APP ratios[146] and ratios increased in AD patients using anticholestorol drugs.[147] Thus in Alzheimer's disease, a peripheral marker, correlating with the severity of the disease, but also in relation with known pathological changes is found. More research is necessary in a large cohort of patients, carefully controlled with patients with other neurological diseases to improve the specificity and sensitivity of this set of markers. If successful, this marker can be used in diagnosis of Alzheimer's disease.

4.3.2 Epilepsy

Peripheral benzodiazepine receptors

Benzodiazapines are used to treat status epilepticus and is thought to activate the GABAergic neurotransmitter system, through interaction with benzodiazepine receptors (part of the ionotropic GABA-A receptor). Peripheral benzodiazepine receptors, not coupled to GABA receptors, might have a role in epilepsy and in antiepileptic drug action.[148] Leukocytes express peripheral benzodiazepine receptors (PBR). Anticonvulsant drugs like diazepam, carbamazepine and phenobarbital occupy these receptors at normal therapeutic range.[149] Chronic use of these drugs increased the expression of PBRs on human leukocytes[150,151] although not consistently,[148] and increased the maximal binding capacity (B_{max})[149] and can therefore be used as a peripheral marker of drug effects on the central nervous system. Drug refractory patients had decreased expression of PBRs.[148] The diazepine binding inhibitor (DBI) is also present in leukocytes, and is increased in untreated epilepsy patients and is only slightly further increased after anticonvulsant drug treatment.[150] The concentration of DBI in plasma is increased in patients with epilepsy, both in adults and pediatric patients and most pronounced in drug refractory patients.[152]

Blood changes as signs of altered glutamate/GABA neurotransmission

The most obvious parameter is the plasma concentrations of various amino acids. Rainesalo *et al*. report normal plasma concentrations of aspartate, glutamate and glycine in HS-TLE and juvenile myoclonic epilepsy (JME) patients.[153] Although contradictory, these authors report also increased levels in plasma of glutamate and other amino acids immediately after seizures, as well as increased plasma glutamate levels in JME patients.[154] These increased levels of plasma glutamate are also found in patients with primary generalized epilepsy, and moreover also in their first degree relatives compared to unrelated controls.[155] In patients with refractory focal epilepsy plasma glutamate level was the same as in control subjects.[154] In epileptic EL mice an increase in plasma glutamate concentrations was found.[156]

As platelets possess high affinity glutamate uptake,[157] and impaired glutamate uptake is involved in the pathogenesis of HS-TLE, it is interesting to measure glutamate uptake in blood platelets. In HS-TLE patients, the affinity of glutamate uptake was increased, but the maximal velocity was lower than in controls.[153] On the other hand, in JME patients, glutamate uptake was unchanged.[153]

Glutamine synthetase, as stated in paragraph 1.2, is the enzyme combining ammonium and glutamate to form glutamine. In HS-TLE patients it was found to be

decreased in brain.[126,127] Peripheral expression of GS might be important for ammonium clearance from the blood, since this can freely pass the blood brain barrier. Plasma levels of glutamine are decreased in JME patients but normal in refractory focal epilepsy patients.[154] The level of urea in blood plasma was decreased and plasma ammonium level was increased in patients with primary generalized epilepsy and in their first degree relatives.[158] Up to now no research has been done regarding levels of GS in blood cells of epilepsy patients. Leukocyte activity of glutamate dehydrogenase, another metabolic enzyme related to glutamate metabolism, was unchanged in patients with primary generalized epilepsy and their first degree relatives.[155]

Not only the glutamate neurotransmitter system is involved in epilepsy, also GABAergic neurotransmission is affected. In blood, platelet GABA uptake was increased and platelet GABA transaminase activity was decreased in children with absence epilepsy.[159] These changes were not present in children with CAE treated with ethosuximide.[159] Platelet GABA-transaminase activity was increased and platelet GABA uptake was decreased in children with JME. No changes were found in these two parameters in refractory localization related epilepsy patients.[160]

Immune system

Immunoglobulins against the AMPA receptor GluR3 are found in a large percentage of patients with Rasmussen encephalitis, but also in more than half of the patients with partial epilepsy[161] or generalized epilepsy[162] and in almost all patients with severe, early onset, intractable ("catastrophic") epilepsy patients.[162]

Changes related to medication

Obviously, systemically given drugs will also have peripheral effects if the appropriate receptors are present. For example, vigabatrin, an inhibitor of GABA–transaminase, increases the GABA concentration in brain. In rats a high correlation is found between GABA-transaminase activity in platelets and brain levels of GABA, suggesting that GABA-transaminase activity is a marker of the efficacy of vigabatrin.[163] In an add-on study of lamotrigine, GABA levels in CSF were specifically increased in patients already treated with vigabatrin, but not in combination with other drugs. CSF levels of glutamate, aspartate and glycine were not affected by add-on therapy with lamotrigine.[164]

AEDs also have side effects related to the normal function of the blood. For example, platelet count, aggregation and ATP release are decreased in children treated with valproate for 6 month.[165] Diazepam, and in smaller degree clonazepam, may down-regulated platelet activation and release of some proinflammatory mediators by stimulated neutrophils.[166] Changes in blood found in idiopathic or symptomatic epileptic children during treatment with vigabatrin indicates that this drug modulates the immune system, especially the cytotoxic cell population.[167]

4.3.3 Amyotrophic lateral sclerosis

Immune system

In ALS a small number of studies report peripheral changes in the immune system.

These include an elevation of plasma TGF1β, which is correlated with the severity of the disease[168] and cellular changes; a higher percentage of activated monocytes/macrophages (which was also shown in patients with Alzheimer's disease) not correlated with disease progression, and an increase in $CD4^+$ T-cells.[169] Serum antibodies (IgG and IgM) were changed as well, IgG was decreased and IgM increased.[169]

Oxidative stress

Related to the idea that oxidative stress is involved in the pathogenesis of ALS, several parameters indicating oxidative stress have been studied in blood, for instance glutathione peroxidase. Extracellular superoxide dismutase and glutathione peroxidase were unchanged in familiar ALS patients with the homozygous mutation in SOD1 (Asp90Ala).[170] However another study in sporadic ALS patients showed that plasma superoxide dismutase activity was increased and glutathione peroxidase was decreased.[171] Products or signs of lipid peroxidation were significantly increased.[171,172]

Excitotoxicity

Plasma amino acids were also studied in ALS patients. Data on blood levels of glutamate in ALS are conflicting. Some studies did not find any change in plasma glutamate levels,[38,173,174] but decreased concentration of other amino acids: valine, isoleucine, leucine, tyrosine and aspartate.[174] Another study found increased levels of glutamate in plasma[175] and yet another study found normal levels in basal plasma, but increased levels after oral glutamate loading.[176] Platelets have a high affinity glutamate uptake.[157] A 43% reduction in platelet glutamate uptake has been found in ALS patients.[177]

5 Aim and outline of this thesis

As described in the preceding paragraphs, peripheral markers of neurological diseases can be of value in diagnosis, patient classification or treatment decisions, and may give clues about the pathogenesis of the disease, if the markers reflect central aberrations. Blood is easy accessible tissue and, because it has different cells types, has multiple suitable parameters.

It is suggested that glutamate neurotoxicity plays a role in a number of neurological diseases. The aim of the research described in this thesis is to identify parameters on different types of blood cells that can serve as peripheral markers in diseases associated with glutamate neurotoxicity. To reach this, I focused on the proteins/enzymes of the glutamate-glutamine cycle, because of the role of this cycle in glutamate neurotoxicity. Next, the found parameters are tested in two neurological diseases, epilepsy and ALS.

Platelets share certain cellular aspects with neurons; particularly, they express neurotransmitter transporters and receptors. Glutamate uptake is similar in platelets

and in brain, although the maximum capacity in platelets is lower.[157] Since the molecular identity of the glutamate transporters on human platelets is yet unknown, this was investigated in *chapter 2*. Also the effect of platelet activation on glutamate uptake was determined. In ALS, spinal cord and cortical areas of motor neuron loss show a decrease in EAAT2 expression.[41,42] Therefore, in *chapter 3*, blood platelets glutamate uptake in ALS patients was compared to healthy controls, as well as the protein expression of EAAT2 and GS on blood platelets.

Blood platelets, as cell-fragments, have no active transcription and translation, therefore, mRNA expression analysis is not possible. This method has advantages; it is possible to study a larger number of genes simultaneously. Leukocytes, another group of blood cells, are nucleated and easy to isolate and store. Therefore, in *chapter 4* the presence of EAATs and GS protein and mRNA in human leukocytes was investigated. Using microarray analysis leukocyte expression profile of genes involved in glutamate neurotransmission was determined in newly diagnosed epilepsy patients compared to gender- and age-matched controls. Several differences in expression level were confirmed using quantitative PCR (qPCR).

To learn more about the nature of the leukocyte expression changes in genes of the glutamate-glutamine cycle, a longitudinal study in epilepsy patients comparing blood- and brain expression would be ideal. As longitudinal studies in humans using both blood and brain is impossible we used an animal model of human TLE, the juvenile pilocarpine model. As the longitudinal expression of proteins of the glutamate-glutamine cycle in the hippocampus in this model is yet unknown, in *chapter 5* this was investigated. Leukocytes were also collected from the same animals during sacrifice. *Chapter 6* describes the analysis of leukocyte GS expression at different time-points after induction of SE.

Finally, the research described in this thesis is summarized and discussed in *chapter 7*.

2

Thrombin-stimulated glutamate uptake in human platelets is predominantly mediated by the glial glutamate transporter EAAT2

G. Hoogland‡, I.W.M. Bos‡, F. Kupper, G. van Willigen, H.A. Spierenburg, O. van Nieuwenhuizen, P.N.E. de Graan

‡These authors contributed equally to this work.

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Abstract

Glutamate toxicity has been implicated in the pathogenesis of various neurological diseases. Glial glutamate transporters play a key role in the regulation of extracellular glutamate levels in the brain by removing glutamate from the extracellular fluid. Since human blood platelets possess an active glutamate uptake system, they have been used as a peripheral model of glutamate transport in the central nervous system (CNS). The present study is aimed at identifying the glutamate transporter on blood platelets, and to asses the influence of platelet activation on glutamate uptake. Platelets from healthy donors showed Na^+-dependent glutamate uptake (K_m 3.5 ± 0.9μM; V_{max} 2.8 ± 0.2 pmol glutamate/75*10^6 platelets/30 min), which could be blocked dose-dependently by the EAAT specific inhibitors DL-threo-E-benzyloxyaspartate (TBOA), L-trans-pyrrolidine-2,4-dicarboxylic acid (tPDC) and high concentrations of the EAAT2 inhibitor dihydrokainate (DHK).

Analysis of platelet homogenates on Western blots showed EAAT2 as the predominant glutamate transporter. Platelet activation by thrombin caused an increase in glutamate uptake, which could be inhibited by TBOA and the EAAT2 inhibitor DHK. Kinetic analysis showed recruitment of new transporters to the membrane. Indeed, Western blot analysis of subcellular fractions revealed that α-granules, which fuse with the membrane upon thrombin stimulation, contained significant EAAT2 immunoreactivity. Inhibition of the second messengers involved in α-granule secretion (protein kinase C, phosphatidylinositol-3-kinase) inhibited thrombin-stimulated uptake, but not basal uptake. These data show that the glial EAAT2 is the predominant glutamate transporter on blood platelets and suggest that thrombin increases glutamate uptake capacity by recruiting new transporters (EAAT2) from α-granules.

Introduction

In the CNS, glutamate transporter proteins are of critical importance for the removal of extracellular glutamate. Low extracellular glutamate concentrations in the synaptic cleft are essential to facilitate fast glutamatergic transmission and to prevent excitotoxicity (for review, see [11]). It is therefore not surprising that reduced glutamate uptake capacity, due to malfunctioning or decreased expression of glutamate transporters, has been implicated in the pathogenesis of various neurological diseases, including cerebral ischemia,[178] amyotrophic lateral sclerosis,[41] and epilepsy.[124]

Five mammalian EAAT isoforms have been cloned and characterized electrophysiologically and pharmacologically: EAAT1 (a.k.a. GLAST),[5,6] EAAT2 (a.k.a. of GLT1),[5,7] EAAT3 (a.k.a. EAAC1),[5,8] EAAT4[9] and EAAT5.[10] EAAT family members display about 50-55% amino acid sequence identity and an almost identical hydrophobicity pattern, suggesting that each transporter exhibits its functional properties on the basis of similar characteristics. In the CNS EAAT1 and EAAT2 are mainly expressed in astrocytes, whereas EAAT3 is highly expressed in neurons of the cortex, hippocampus and caudate-putamen.[179] EAAT4 is highly expressed in cerebellar Purkinje cells with little expression in other brain regions[180] and EAAT5 is expressed predominantly in the retina.[10] Although both neurons and glia contain glutamate transporters, it is generally accepted that the uptake capacity of astrocytes is much higher than that of neurons.[3]

Glutamate transporter subtypes EAAT1-3 are not only expressed in the CNS, but also in various peripheral tissues (such as lung, liver, muscle, and heart (reviewed in [11]). Human blood platelets also contain a high affinity, Na^+-dependent glutamate uptake system, with similar kinetic and pharmacological properties as neurons and glia.[157]

Glutamate transport in blood platelets has been studied as a model for glutamate transport in the CNS, e.g. as a peripheral marker for excitotoxicity in neurodegenerative diseases such as Parkinson's disease,[181] amyotrophic lateral sclerosis,[177] epilepsy[153] and Alzheimer's disease.[182,183] The latter study[183] suggest that EAAT1, 2 and 3 are expressed on human blood platelets.

The influence of platelet activation, e.g. by thrombin, on glutamate uptake is unknown. Thrombin is a potent, physiological platelet activator, responsible for the final steps in blood coagulation and thrombus formation. Full platelet activation by thrombin leads to the fusion of α-granules with the platelet membrane. Heijnen *et al*. (1997)[184] have shown that thrombin-induced platelet activation is accompanied by an increase in glucose uptake capacity, caused by recruitment of glucose transporter GLUT-3 from α-granules to the platelet membrane.

The aim of this study was to identify the EAAT subtype on human blood platelets by probing Western blots with subtype specific antisera. Subsequently, we determined the effect of thrombin activation on platelet glutamate uptake and characterized the kinetics and pharmacology of glutamate uptake in the presence and absence of thrombin.

Materials and Methods

Isolation of platelet-rich plasma

Blood was collected by venipuncture from healthy donors with informed consent and was anticoagulated with 0.1 vol of 0.13 M sodium citrate. Platelets were isolated as published earlier[185] with minor modifications. Briefly, platelet-rich plasma (PRP) was obtained by centrifugation of whole blood at 300xg for 15 min at room temperature and then acidified with 0.1 vol Acid-Citric acid-Dextrose (ACD, 85 mM tri-sodium citrate, 71 mM citric acid, 100 mM glucose). Platelets were then isolated by gel-filtration (according to Akkerman *et al.* (1978)[186]) or centrifugation as described below. All steps were carried out in plastic tubes and in calcium-free buffers to prevent platelet activation.

Platelet isolation by gel filtration

Acidified PRP was loaded on a Sepharose 2B (Amersham) column equilibrated with a Ca^{2+}-free Tyrode buffer (137 mM NaCl, 2.68 mM KCl, 0.42 mM NaH_2PO_4, 1.7 mM $MgCl_2$, 11.9 mM $NaHCO_3$, pH 7.25) containing 5 mM glucose and 0.2% Bovine Serum Albumin (BSA).[186] The collected platelet fraction was acidified by adding 0.1 vol ACD. After centrifugation (900xg for 15 min at room temperature) the platelet pellet was resuspended in Hepes-Tyrode buffer (145 mM NaCl, 5 mM KCl, 0.5 mM Na_2HPO_4, 1 mM $MgSO_4$, 10 mM Hepes, pH 7.2) containing 5 mM glucose, and the platelet concentration was determined in a platelet counter. Finally, the platelet suspension was dissolved in sample buffer (final concentration: 0.001 w/v % bromophenolblue, 5% v/v β-mercaptoethanol, 10% v/v glycerol, 2% w/v SDS, 62.5 mM Tris-HCl, pH 6.8), and analysed by immunoblotting. As positive control for EAAT2 immunostaining, two different HEK cell lines stably expressing EAAT2, were used (kindly provided by L.A. Wisman[187]). Cells were homogenised in 0.5% v/v TritonX100, 1mM EGTA, 1mM EDTA, 1 mM Na_3VO_4 and 1 mM phenylmethylsulfonyl fluoride. After protein determination the samples were dissolved in sample buffer.

For immunoblot analysis of subcellular fractions, we used two independent preparations of plasma membrane and granule fractions, isolated from platelets of multiple donors as published earlier.[188]

Platelet isolation by centrifugation

The PRP was centrifuged (900xg for 15 min at room temperature), where after the platelet pellet was washed and resuspended in Tris-citrate buffer (112 mM NaCl, 4 mM KCl, 1.1 mM KH_2PO_4, 1.1 mM $MgSO_4$, 11 mM Na_3Citrate, 25 mM Tris-HCl, pH 7.0) containing 10 mM glucose and further used for glutamate uptake studies.

Glutamate uptake

Specific Na^+-dependent [^{3}H]-glutamate uptake was measured as described by Mangano and Schwarzc (1981).[157] Briefly, the isolated platelets were diluted to

$150*10^6$ platelets/ml in either Na^+ or no-sodium-containing Tris-citrate buffer (sodium chloride substituted by equimolar choline-chloride, sodium-citrate substituted by equimolar potassium-citrate). Next, 500 µl aliquots were preincubated (20 min at 37°C) and further incubated (10 min at 37°C) either in the absence (basal uptake) or presence of thrombin (human α-thrombin, final concentration 0.25 or 0.5 U/ml; Kordia), ADP (final concentration 10 µM; Roche) L-trans-pyrrolidine-2,4-dicarboxylic acid (tPDC, final concentration 2, 10, 50 or 250 µM; Tocris), DL-threo-E-benzyloxyaspartate (TBOA, final concentration 1, 6, 36 or 216 µM; Tocris), or dihydrokainate (DHK, final concentrations 0.1 to 1000 µM; Tocris). In some experiments protein kinase C (PKC) inhibitor peptide 19-27 (final concentration 10 µM; Calbiochem) or phosphatidylinositol 3-kinase (PI_3K) inhibitor LY294002 (final concentration 50 µM; Tocris) was added 2 min prior to the addition of thrombin. The uptake assay was then initiated by adding [^{3}H]-glutamate (final concentration 1 µM; 0.4 µCi /sample, specific activity 42 Ci/mmol, ICN Pharmaceuticals) and incubated for 30 min at 37°C. In initial experiments linearity of the uptake was confirmed by incubating the samples 10, 20 and 30 minutes with [^{3}H]-glutamate at 37˚C. Uptake was terminated by adding 3 ml ice-cold Na^+-Tris-citrate buffer containing 1 mM glutamate. Platelets were harvested using a Brandel cell harvester on Whatman glass fiber paper GF/B under vacuum. Filters were rinsed four times with 3 ml ice-cold Na^+-Tris-citrate buffer containing 1 mM L-glutamate. Radioactivity on the filters was quantified by liquid scintillation counting. Glutamate uptake was expressed as pmol glutamate/$75*10^6$ platelets/30 minutes or as percentage of basal, Na^+-dependent uptake. IC_{50} was determined using non-linear regression, one site inhibition (GraphPad Prism, GraphPad Software Inc.). K_i was calculated with the Cheng-Prusoff equation[189] and the K_m for glutamate uptake as described below.

Kinetic analysis

Platelets were preincubated in either Na^+ or choline-containing Tris-citrate buffer ($75*10^6$ platelets/0.5 ml) for 20 min at 37°C, followed by either an additional incubation of 10 minutes (basal uptake) or addition of thrombin (0.5 U/ml) and incubation for an additional 10 min. Next, platelets were incubated in the presence of 0.05, 0.1, 1, 3, 5, 10 or 30 µM L-glutamate plus 0.4 µCi [^{3}H]-glutamate per sample, followed by determination of [^{3}H]-glutamate uptake as described above. Glutamate concentrations were corrected for the radioactive glutamate added. K_m and V_{max} were represented in an Eadie-Hofstee plot and calculated by non-linear regression (GraphPad Prism, GraphPad Software Inc.).

Immunoblotting

Aliquots of platelets ($20*10^6$ platelets), plasma membrane fraction (50 µg protein), granule fraction (50 µg protein) and human cortex homogenate (1 or 10 µg) or HEK cells (10 µg) were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (11% v/v polyacrylamide gels) and transferred to polyvinylidene difluoride (PVDF) membranes (Amersham) by electroblotting for 1.1 hr at 400 mA. Membranes were blocked (1 hr) in 50 mM Tris-buffered saline containing 0.1% v/v

Tween 20 (TBSt) at room temperature, supplemented with 5% w/v lowfat dry milk, and 10% w/v Bovine Serum Albumin (BSA) for EAAT1 and 2, and 5% w/v BSA for EAAT3. Membranes were then incubated (overnight at 4°C) with GLAST antibody (1:5,000; 10% w/v BSA, guinea pig polyclonal, Chemicon), GLT1 antibody (1:2,000; 10% w/v BSA, guinea pig polyclonal, Chemicon or 1: 200, 5% w/v BSA, mouse monoclonal, Novocastra) or EAAC1 antibody (1:500; 5% w/v BSA, rabbit polyclonal, Biotrend). The polyclonal antibodies are directed against rat peptides, but show cross-reactivity with human EAAT1, 2, or 3 respectively. Membranes were washed, and incubated for 1 hr with alkaline phosphatase-conjugated goat anti guinea pig IgG, goat anti rabbit IgG (both 1:2,000; Jackson Immuno Research, 10% w/v added for EAAT1, 2, and 5% w/v BSA for EAAT3) or goat anti mouse IgG (1:5000, Promega). Finally, immunoreactive proteins were stained with enhanced chemifluorescence substrate (Amersham) and visualized by a Fluor-S MultiImager (Biorad). As a control for antibody specificity, we omitted the primary antibody in the immunoblot procedure. Protein loading and blotting was controlled by Ponceau-S staining.

Results

Transporter-mediated glutamate uptake in platelets

Uptake in the absence of Na^+ resulted in the accumulation of about 29% of the glutamate taken up in the presence of Na^+, resulting in a Na^+-dependent glutamate uptake of 0.36 ± 0.08 pmol/75*10^6 platelets/30 min (n=9, fig. 1a). Uptake was linear at 30 minutes of incubation time. The kinetic constants K_m and V_{max} of platelet glutamate uptake were determined and graphically depicted in an Eadie-Hofstee plot (fig. 1b). Calculation of these values by non-linear regression fit showed a K_m of 3.5 ± 0.9 µM and a V_{max} of 2.8 ± 0.2 pmol/75*10^6 platelets/30 min (n=9), similar to values obtained by Mangano and Schwarz (1981).[157] Glutamate transport inhibitor tPDC specifically and dose-dependently reduced the Na^+-dependent glutamate uptake, resulting in 70% (2 µM), 38% (10 µM), 20% (50 µM) and 1% (250 µM) of the control uptake value (n=4, fig. 2a). IC_{50} is approximated at 15.8 µM and the corresponding K_i is 12.3 µM. TBOA, a non-transportable inhibitor dose-dependently inhibited Na^+-dependent glutamate uptake to 70% (1 µM), 28% (6 µM), 10% (36 µM) and 3% (216 µM) of the control uptake value (n=9, fig. 2b). IC_{50} is approximated at 2.2 µM and the corresponding K_i is 1.7 µM. None of the inhibitors influenced the aspecific, Na^+-independent glutamate uptake (data not shown).

The results of the three experiments described above strongly suggest the presence of EAATs on blood platelets. We therefore analyzed the protein expression of EAAT1, 2 and 3 by immunoblotting. Homogenates from human cortical brain tissue were used as positive control. We were unable to detect EAAT1, or EAAT3 immunoreactivity (IR) in platelets. Positive controls, using brain cortex, showed a single IR band at the appropriate apparent molecular weights.[124] Antibodies to EAAT2, which is the major glial glutamate transporter and is responsible for 90% of the glutamate transport in the CNS[190] showed a single immunoreactive band with an apparent molecular weight of ~79 kDa (fig. 2c, lane 6,7), migrating at the same apparent molecular weight as

EAAT2 in two HEK cell lines stably expressing EAAT2[187] (fig. 2c, lane 4,5). EAAT2 IR in brain homogenates migrates at a slightly lower apparent molecular weight of ~73 kDa (fig. 2c, lane 1,2). The identity of the EAAT2 band in platelet homogenates was further confirmed by using a monoclonal EAAT2 antibody (fig. 2c, lane 8). Omission of the primary antibody abolished all staining (fig. 2c, lanes 9-14).

Thrombin-stimulated glutamate uptake in platelets

Platelets are important in blood coagulation. Thrombin activates platelets, which results in thrombus formation. Na^+-dependent glutamate uptake was 5-fold increased when platelets were stimulated with a low dose of thrombin (0.25 U/ml, n=18) and 9-fold increased with a dose of 0.5 U/ml (n=12, fig. 3a). The thrombin-induced increase in glutamate uptake was TBOA sensitive. In the presence of 36 µM TBOA, thrombin stimulated uptake was reduced to 9.3 % (n=9, data not shown). DHK, a selective inhibitor of EAAT2,[5,191,192] inhibited basal glutamate in a dose-dependent manner, but only in high concentrations. Thrombin-stimulated uptake was inhibited dose-dependently with a IC_{50} approximated at 105.5 µM and the corresponding K_i of 82.0 µM (fig. 3b).

Platelet activation induced recruitment of EAAT2 from α-granules

To determine whether the thrombin-induced increase in platelet glutamate uptake involves α-granule secretion, we performed a series of experiments similar to those used by Heijnen et al. (1997).[184] These authors showed that thrombin activation of platelets is accompanied by an increase in glucose uptake, which is caused by recruitment of glucose transporter GLUT-3 from secreted α-granules.

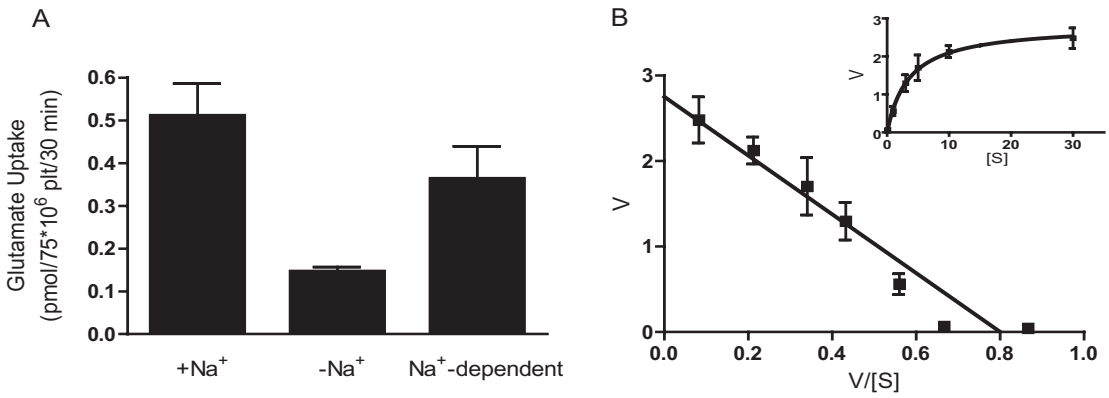

Figure 1. *Na^+-dependent glutamate uptake.*
*Na^+-dependency was established by performing the glutamate uptake assay in the presence of Na^+ ($+Na^+$) and absence of Na^+ ($-Na^+$). The amount of Na^+-dependent uptake was calculated by subtracting uptake in the presence and absence of Na^+ (A). Kinetic analysis of glutamate uptake is depicted in an Eadie-Hofstee plot (B). Platelets were incubated with various concentrations of glutamate. Km (3.5 ± 0.9 µM) and Vmax (2.8 ± 0.2 pmol/75*106 plt/30min) were calculated by non-linear regression (GraphPad Prism). Inset: saturation curve of platelet glutamate uptake. Uptake velocity (V) is expressed as pmol/75*106 plt/30 min and substrate concentration (S) as µM. Values represent mean ± SEM of 9 donors.*

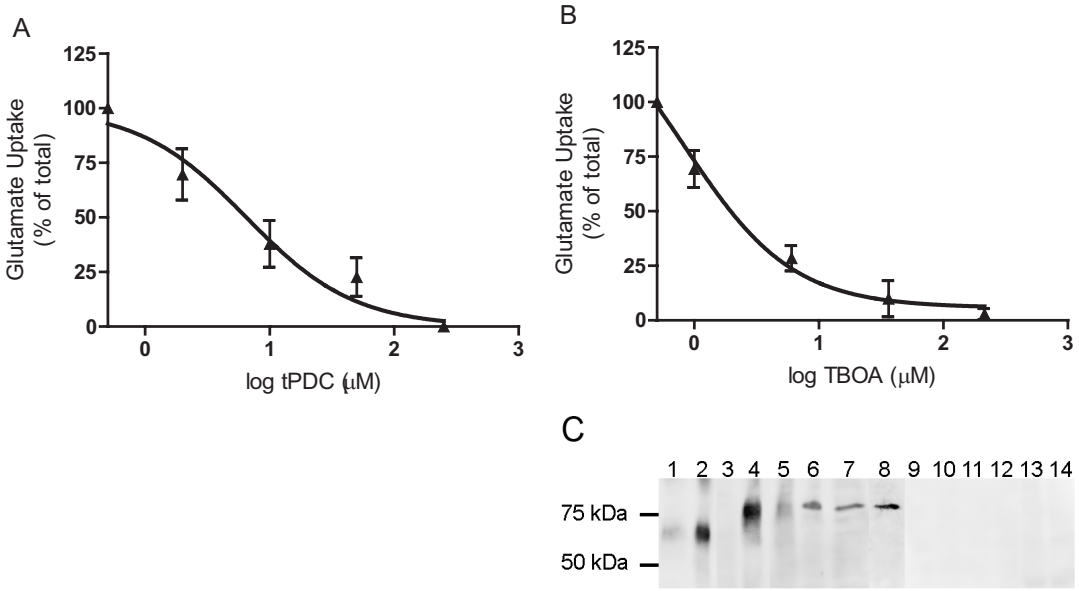

Figure 2. *Glutamate transporter EAAT2 expression.*
Platelets were preincubated with different concentrations of tPDC (A) and TBOA (B). This resulted in a dose-dependent inhibition of Na^+-dependent glutamate uptake. Data are expressed as percentage of total glutamate uptake and concentration of inhibitor in μM (log) and represent mean ± SEM of 4-9 donors. Curves were fitted using GraphPad Prism, one site competition. C: EAAT2 immunoreactivity was detected in platelet lysates on Western blots with two antibodies against EAAT2 (lane 6&7 polyclonal Ab (Chemicon); lane 8, monoclonal Ab (Novocastra; ($20*10^6$ platelets/lane)). As positive controls were used: human cortex homogenate (lane 1 (1μg protein/lane) and 2 (10 ug protein/lane)) and HEK cell lines stably expressing EAAT2 (lane 3; wild type HEK cell (10 μg protein/lane), lane 4 & 5; stable EAAT2 expressing HEK cells lines (10 μg protein/lane)). Antibody specificity was determined by omitting the primary antibody from the staining procedure (lanes 9-14; 9,10 human cortex, 11 HEK wild type, 12 EAAT2 HEK, 13,14 platelets).

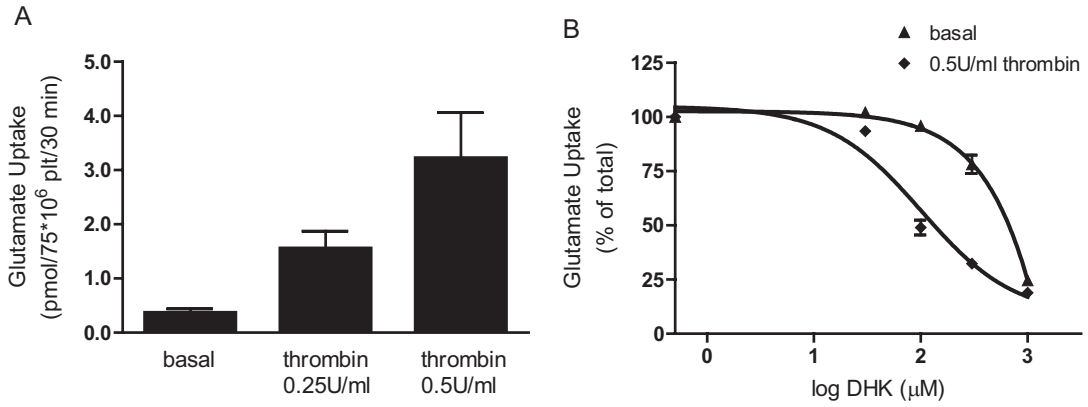

Figure 3. *Thrombin stimulated glutamate uptake.*
Preincubation of platelets with thrombin (0.25 and 0.5 U/ml) prior to the glutamate uptake assay resulted in an increase of Na^+-dependent glutamate uptake (A), which could be inhibited by preincubating platelets with both thrombin (0.5 U/ml) and DHK (B). Data are expressed as mean ± SEM (A) or as percentage of total glutamate uptake (B), and are obtained from 12-18 donors. Curves were fitted using GraphPad Prism, one site inhibition.

First, we treated platelets with 10 µM ADP causing characteristic changes in platelet shape, i.e., centralisation of α-granules and the formation of pseudopodal extensions, but no secretion of α-granules.[184] This did not affect glutamate uptake (n=9, fig. 4a). Subsequently, we determined uptake kinetics in the presence or absence of thrombin. As illustrated in the saturation curve (n=9, fig. 4b), stimulation of platelets with thrombin (0.5 U/ml) increased the V_{max} approximately 9 times (i.e. indicating a denser population), compared to unstimulated platelets. Next, we probed immunoblots loaded with subcellular fractions of human platelets with the EAAT2 antibody (equal amounts of protein per fraction, fig. 4c). The plasma membrane and the granule fraction both showed a single EAAT2 IR band, which was most prominent in the granule fraction. Finally, since granule secretion requires second messenger pathways

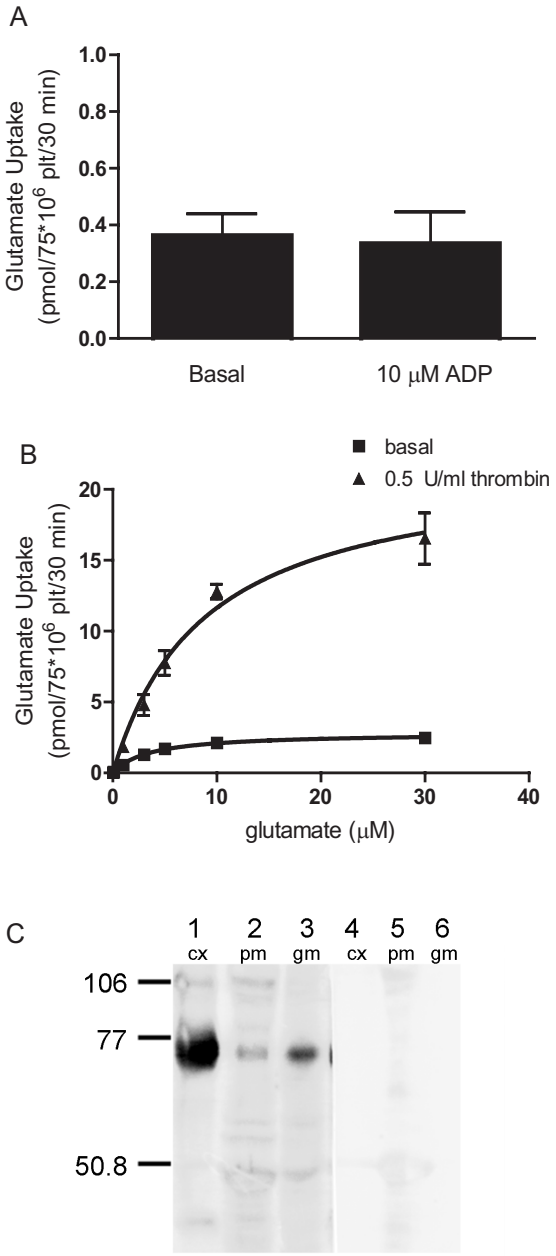

Figure 4. *Glutamate transporters are localized in α-granules.*
*(A) Platelets were preincubated with ADP (10 µM), a concentration known to induce shape changes, but no granule secretion. Compared to basal glutamate uptake, ADP treatment did not increase glutamate uptake. (B) After preincubation with thrombin (0.5 U/ml), the kinetics of glutamate uptake were analyzed by incubating platelets with different glutamate concentrations. Results are depicted as saturation curve of platelet glutamate uptake. Uptake velocity (V) is expressed as pmol/75*10^6 plt/30 min and substrate concentration (S) as µM. Values represent mean ± SEM of 9 donors (B). Thrombin stimulation led to an increase in V_{max}, indicating an increased number of transporters due to thrombin stimulation. (C) Immunoblotting showed EAAT2 immunoreactivity in the platelet membrane fraction (pm; lane 2, 50 µg protein/lane) and the α-granule fraction (gm; lane 3, 50 µg protein/lane). Human cortex (cx; lane 1, 1 µg protein/lane) was used as positive control. Antibody specificity was determined by omitting the primary antibody from the staining procedure (lanes 4-6).*

of the competitive PKC inhibitor PKC_{19-27} (10 µM), thrombin-stimulated glutamate uptake (0.5 U/ml) was inhibited by 57% (n=12, fig. 5a). This inhibitory effect was specific for thrombin-stimulated glutamate uptake, since PKC_{19-27} (10 µM) did not affect basal glutamate uptake. PI_3K inhibitor LY294002 (50 µM) inhibited thrombin-stimulated glutamate uptake (0.25 U/ml) by about 30% without affecting basal glutamate uptake (n=12, fig. 5b).

Discussion

The presence of a glutamate transport system on blood platelets has been known for many years,[157] and the tissue distribution of the 5 cloned glutamate transporters has been studied in detail (for review see [11]). However, the EAAT subtype responsible for platelet glutamate uptake has not been identified. Here we confirm the presence of a Na^+-dependent glutamate uptake system (V_{max} 2.8 pmol/75*106 plts/30 min; K_m 3.5 µM) on human blood platelets, which can be inhibited by the specific glutamate uptake inhibitors t-PDC and TBOA. Our V_{max} (recalculated in pmol/mg protein/10min: 7.3± 0.5) and K_m values are similar to those reported by Mangano and Schwarcz (1981),[157] but are almost 10-fold lower than reported by Ferrarese *et al*. (1999,2000).[181,182] This difference is probably caused by the fact that the latter group uses frozen and re-thawn platelets for the uptake assay. In our hands a freeze-thaw cycle causes severe platelet damage.

Using two subtype specific antibodies we identified EAAT2 as a single immunoreactive band in human platelet homogenates, migrating at the same apparent molecular weight as EAAT2 in HEK cells stably transfected with EAAT2. Basal uptake could only be inhibited by high concentration of DHK, the selective inhibitor of EAAT2.[5,191,192] Using subtype specific antibodies we were unable to detect EAAT1 and EAAT3, the other two glutamate transporters which are widely distributed outside the CNS. However, we cannot rule out low levels of expression of EAAT1 and EAAT3.[183] We conclude that under basal uptake conditions EAAT2 contributes to

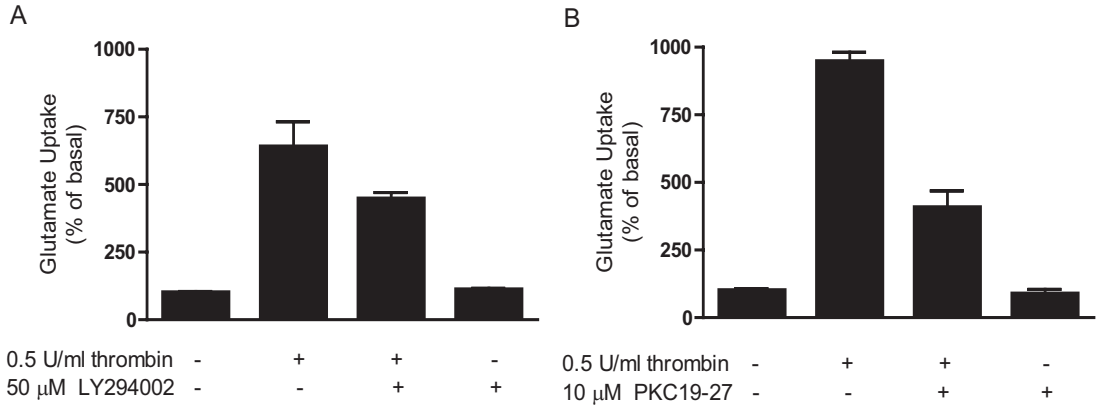

Figure 5. *PKC and PI_3-kinase inhibition reduced thrombin-stimulated glutamate uptake. Prior to the glutamate uptake assay, platelets were preincubated with thrombin (0.5U/ml), PKC inhibitor peptide 19-27 (10 µM) or PI_3K inhibitor LY294002 (50 µM) or a combination of thrombin and inhibitor (fig. A and B respectively). The increase in glutamate uptake upon stimulation is expressed relative to glutamate uptake values of unstimulated platelets. The values represent the mean ± SEM from 12 donors.*

platelet glutamate uptake, but possibly other transporters are involved as well.
Activation of human platelets with thrombin (0.5 U/ml) caused a 9-fold increase in Na^+-dependent uptake and had no effect on Na^+-independent uptake. The increase can be inhibited by DHK and is thus mediated by EAAT2. DHK inhibits thrombin-stimulated transport with an K_i of 82 µM, which is slightly higher than reported in literature.[5,191,192] Several lines of evidence indicate that the rapid elevation in glutamate uptake is associated with α-granule secretion. Firstly, stimulation with 10 µM ADP, a concentration that is known to induce shape changes but no significant α-granule secretion, had no effect on the glutamate uptake capacity. Secondly, the uptake kinetics after thrombin stimulation indicates an increase in transporter density (large increase in V_{max}). Thirdly, EAAT2 immunoreactivity was predominantly found in α-granules isolated from unstimulated platelets. Finally, blockade of the second messenger pathways involved in thrombin receptor activation and α-granule secretion (PI_3K and PKC) reduced the thrombin-induced increase in glutamate uptake. Taken together, these data strongly suggest that thrombin stimulates glutamate transport via translocation of EAAT2 from α-granules to the cell surface. A similar translocation mechanism from α-granules to the platelet membrane has been described for glucose transporter GLUT-3 during thrombin-stimulated glucose transport.[184]
The functional significance of thrombin-stimulated glutamate uptake by blood platelets is unknown. Thrombin, produced at sites of tissue damage, activates platelets, and thereby catalysis thrombus formation. An increase in glucose uptake upon platelet activation was previously suggested to relate to the high-energy demands of this process.[184] Glutamate may serve as metabolic fuel for the tricarboxylic acid cycle, and thus also contributes to the energy provision in platelets. It was shown previously that thrombin increases the glutamine to glutamate conversion in platelets, thereby contributing to the energy supply.[195] In addition, platelet glutamate uptake may be important to neutralize endogenous ammonia through the formation of glutamine catalyzed by the enzyme glutamine synthetase.[196]
Although the precise function of platelet glutamate uptake remains to be determined, the expression of functional glial EAAT2 in blood platelets opens the possibility to study glutamate transporter properties in patients with neurological disorders.

Acknowledgements

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3

Increased glutamine synthetase but normal EAAT2 expression in platelets of ALS patients

I.W.M. Bos, G. Hoogland, C.F.Meine Jansen, G. van Willigen, H.A. Spierenburg, L.H. van den Berg, P.N.E. de Graan

Submitted for publication

Abstract

Background: Amyotrophic lateral sclerosis is a fatal neurodegenerative disease and glutamate excitotoxicity has been implicated in its pathogenesis. Platelets contain a glutamate uptake system and express components of the glutamate-glutamine cycle, such as the predominant glial excitatory amino acid transporter 2 (EAAT2). In several neurological diseases platelets have proven to be systemic markers for the disease. *Methods:* We compared properties of key components of the glutamate-glutamine cycle in blood platelets of ALS patients and healthy controls. Platelets were analyzed for ^{3}H-glutamate uptake in the presence or absence of thrombin and for EAAT2 and glutamine synthetase protein expression by Western blotting. *Results:* Platelets of ALS patients showed a 34% increase in expression of glutamine synthetase, but normal expression of glutamate transporter EAAT2. Glutamate uptake in resting or thrombin-stimulated platelets did not differ significantly between platelets from ALS patients and controls. Thrombin-stimulation resulted in about a 7-fold increase in glutamate uptake. *Conclusions:* Our data suggest that glutamine synthetase may be a peripheral marker of ALS and encourage further investigation into the role of this enzyme in ALS.

Introduction

Amyotrophic lateral sclerosis (ALS) is an adult onset and fatal neurodegenerative disease characterized by selective upper and lower motor neuron degeneration. One of the proposed pathogenic mechanisms is glutamate excitotoxicity, either caused directly by excessive extracellular glutamate or indirectly by the compromised energy status of the neurons.[197] Riluzole, the only know drug slowing disease progression, can inhibit glutamate release[198] and increase glutamate uptake.[199,200] Key components of the glutamate-glutamine cycle, excitatory amino acid transporters (EAATs) and glutamine synthetase (GS, E.C. 6.3.1.2) are essential for glutamatergic transmission and important to prevent excitotoxicity.[2]

Reduced GS expression has been implicated in the pathogenesis of temporal lobe epilepsy,[126,127] but no data are available on GS expression in ALS patients. GS activity and expression appear to be unaffected in the spinal cord of the ALS mouse model G93A/SOD-1.[201] Post-mortem brain tissue of ALS patients showed a major loss of glutamate uptake, accompanied by a marked decrease in expression of the major glial glutamate transporter EAAT2 protein.[3,40-42,44] Altered splicing of EAAT2 is thought to play an important role in the pathology of ALS,[43,44] but the precise role of aberrant forms of EAAT2 in ALS needs to be further investigated.[45-50] As an alternative for post mortem studies, patient blood platelets were analyzed in several neurodegenerative diseases, such as Alzheimer's disease.[138] Platelets contain a high affinity, Na^+-dependent glutamate uptake system[157] and express EAAT1 and EAAT3[183] and EAAT2.[202] Thrombin activation of platelets increases glutamate transport by translocation of EAAT2 to the outer membrane.[202] Platelet glutamate uptake was studied in various neurodegenerative diseases including Huntington, Alzheimer, Parkinson and ALS.[153,177,181,203-205] Ferrarese *et al.*[177] reported a 43% decrease in platelet glutamate uptake in ALS patients.

In the present study we analyzed blood platelets of ALS patients and healthy donor controls in search for blood markers for ALS. We determined expression levels of GS and EAAT2 protein by Western blotting and [^{3}H]-glutamate uptake in the presence and absence of thrombin.

Materials and Methods

Patients

Included in this study were 35 ALS patients and 34 healthy donors (control subjects). A detailed description of patients and controls is given in table 1. Patients were diagnosed with definite or probable ALS according to the El Escorial criteria.[206] Donors that used NSAIDs were excluded. ALS patients had a mean duration of disease of 20 months and all but four were on riluzole treatment. Twenty-nine patients had a spinal site of onset, and six had a bulbar site of onset. The mean age in the ALS group (57 years; range 36-78; 14F, 21M) was higher than in the healthy donors (37

Table 1: Summary of clinical data of patients and controls.

	ALS	CTR
n	35	34
Sex	14	17
Female	21	16
Male		
Mean age (years)	57	37
Duration of disease (months)	20	n.a.
Site of onset	6	n.a.
bulbar	29	
spinal		
Riluzole treatment	31	n.a.

CTR=healthy donor (control), ALS= amyotrophic lateral sclerosis, n.a. = not applicable

years; range 23-57; $p<0.001$), but no differences were found in gender composition (ALS 14F, 21M, healthy donors: 17F, 16M). Vital capacity was measured at the day of blood collection. The study was approved by the medical ethics review board of the University Medical Center in Utrecht.

Platelet isolation

Blood was collected by venipuncture in 0.1 volume of 3.8% w/v Na_3Citrate in plastic containers. Within 4 hrs after collection, the blood was centrifuged for 15 min at 300xg at room temperature (RT) in a swing-out rotor to obtain platelet-rich plasma (PRP). Then, 0.1 volume of Acid-Citric acid-Dextrose (ACD; 85 mM Na_3Citrate, 71 mM citric acid, 100 mM glucose) was added to the PRP and platelets were purified by gel-filtration on a sepharose 2B column equilibrated with Tyrode buffer (137 mM NaCl, 2.68 mM KCl, 0.42 M Na_2HPO4, 1.7 mM $MgCl_2$, 11.9 mM$NaHCO_3$, 0.1% w/v glucose, 0.2% w/v bovine serum albumin (BSA), pH 7.25). The platelet fraction was acidified with 0.1 vol of ACD and concentrated by centrifugation for 15 min at 1000xg at RT. For Western blot analysis, the platelet pellet was resuspended in Hepes-Tyrode (HT) buffer (145 mM NaCl, 5 mM KCl, 0.5 mM Na_2HPO_4, 1.0 mM $MgSO_4$, 10 mM HEPES and 0.1% glucose, pH 7.25), counted, diluted in sample buffer (final concentration (f.c.) 0.001% w/v bromophenolblue, 5% v/v β-mercaptoethanol, 10% v/v glycerol, 2% w/v SDS, 62.5 mM Tris-HCl, pH 6.8) and stored at -20°. For glutamate uptake, the pellet was resuspended in sodium-Tris-citrate buffer (112.8 mM NaCl, 4.5 mM KCl, 1.1 mM $MgSO_4$, 1.1 mM KH_2PO_4, 25 mM Tris and 11 mM Na_3Citrate, 0.2% glucose, pH 7.0), counted and immediately used for uptake studies as described below. Platelet GS enzyme activity could not be determined in these samples, because there was insufficient material per patient.

Glutamate uptake

To examine glutamate uptake the method described by Mangano and Schwarcz (1981)[157] was used with minor modifications. Briefly, the isolated platelets were diluted to $150*10^6$ platelets/ml in either Na^+ or no-sodium containing Tris-citrate buffer (sodium chloride substituted by equimolar choline-chloride, sodium-citrate substituted by equimolar potassium-citrate). Next, 500 µl aliquots were preincubated (20 min at 37˚C) and further incubated (10 min at 37˚C) either in the absence (control) or presence of thrombin (human α-thrombin, final concentration 0.25 U/ml or 0.5 U/ml, Kordia) or ADP (final concentration 10µM, Roche). The uptake assay was then initiated by adding [^{3}H]-glutamate (final concentration 1 µM; 0.4 µCi / sample, specific activity 42 Ci/mmol ICN Pharmaceuticals). After 30 min incubation at 37°C, uptake was terminated by adding 3 ml of ice-cold sodium-Tris-citrate buffer pH7.0 containing 1 mM glutamate. Platelets were harvested using a Brandel cell harvester M24R on Whatman glass fibre paper GF/B under vacuum. Filters were rinsed four times with 3 ml ice-cold Tris-citrate buffer containing 1 mM L-glutamate. Radioactivity on the filters was quantified by liquid scintillation counting. All determinations were performed in triplicate. Net high-affinity uptake was calculated by subtracting sodium-free samples from sodium-containing samples and was expressed as pmol/$75*10^6$ platelets/30min.

Western blot analysis

Primary antibodies used in Western blot analysis were polyclonal goat anti-EAAT2 (Chemicon International), and monoclonal mouse anti-GS (Transduction Laboratories).

Platelet lysates ($10*10^6$ platelets per lane) were separated by 11% SDS-PAGE and transferred by semi-dry electroblotting for 1.1 hr at 400 mA to polyvinylidene difluoride membranes (PVDF, Amersham). After cutting the membrane at approximately 60 kDa, the lower half was incubated with GS and the upper half with EAAT2 antibodies. Membranes were blocked for 1 hr at RT with 5% w/v lowfat dry milk in 50mM Tris-buffered saline containing 0.1% v/v Tween-20 (TBSt) and BSA (1% w/v for anti-GS and 10% w/v for anti-EAAT2). Membranes were incubated with primary antibody overnight at 4°C (1:2,000, 10% w/v BSA in TBSt for anti-EAAT2; 1:1,000 (0.25µg/ml) in TBSt for anti-GS), washed and incubated with alkaline phosphatase conjugated secondary antibody for 1 hr at RT (1:2,000 Goat-anti-Guinea Pig IgG, 10% w/v BSA in TBSt or 1:5000 Goat-anti-Mouse IgG in TBSt respectively). Subsequently, membranes were washed and incubated with enhanced chemofluorescence substrate (ECF, Amersham), visualized using a Fluor-S MultiImager (BioRad), and fluorescence was quantified with the Quantity One software package (BioRad).

Statistics

Data are expressed as mean ± SEM. Data were analyzed using Student's t-test or Pearson's correlations using the SPSS software package. P-values <0.05 were considered significant.

Results

Glutamate uptake

Glutamate uptake (fig. 1) in blood platelets of 25 healthy donors in the presence of sodium was 0.50 ± 0.06 and non-specific uptake (sodium free samples) was 0.15 ± 0.01 pmol/75*10^6 plts/30 min. Na^+-dependent uptake in healthy donors was 0.35 ± 0.06 pmol/75*10^6 plts/30 min. In ALS patients (n=32), uptake in the presence of sodium was 0.63 ± 0.07 and 0.19 ± 0.02 pmol/75*10^6 plts/30 min in the absence of sodium. Na^+-dependent uptake in ALS patients was 0.44 ± 0.06 pmol/75*10^6 plts/30 min, which did not significantly differ from healthy donors.

Thrombin stimulation (fig. 2) increased Na^+-dependent uptake (compared to basal) via translocation of predominantly EAAT2[202] to 532 ± 70% (0.25 U, n=25) and to 837 ± 107% (0.5 U, n=18) in platelets from healthy donors and in platelets from ALS patient to 395 ± 49% (0.25 U, n=32) and to 683 ± 77 % (0.5U, n=19, mean ± SEM). In

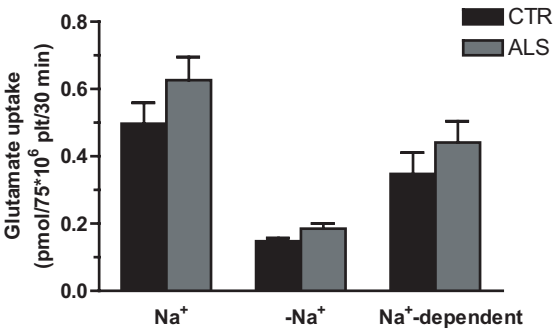

Figure 1. *Sodium dependency of glutamate uptake in blood platelets from ALS patients and healthy controls. As calculated by subtracting Na^+-free samples ($-Na^+$) from Na^+-containing samples (Na^+) most of the glutamate uptake is Na^+-dependent. No difference in glutamate uptake was found between healthy donors (CTR) and ALS patients. (ALS).*

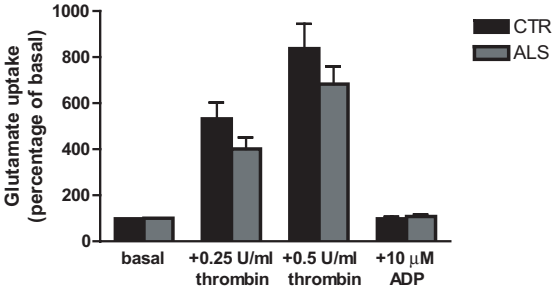

Figure 2. *Effect of activation of blood platelets on Na+-dependent glutamate uptake. Thrombin dose-dependently stimulated Na^+-dependent glutamate uptake, whereas ADP did not. No difference in thrombin stimulated glutamate uptake was found between healthy donors (CTR) and ALS patients (ALS).*

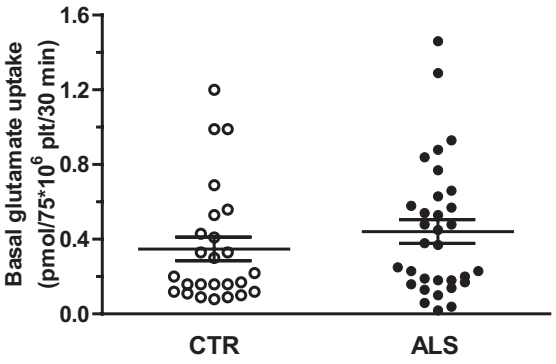

Figure 3. *Scatterplot of individual donors of Na^+-dependent glutamate uptake by blood platelets. The horizontal line indicates the mean ± SEM. No significant difference was observed between healthy donors (CTR) and ALS patients (ALS).*

contrast, ADP did not affect Na^+-dependent glutamate uptake in platelets of healthy donors (98 ± 9%, n=10) or ALS patients (108 ± 8 %, n=13). Neither the absolute amount (data not shown) nor the percentage of increase induced by thrombin or ADP stimulation, were significantly different in platelets of healthy donors and ALS patients.

Variation between donors

The levels of platelet glutamate uptake varied considerably between subjects (fig. 3). This seems not to be a consequence of the large age-range of healthy donors and ALS patients, as statistical analysis showed no correlation between glutamate uptake and age. Differences in quality of the isolated platelets e.g. activation during the isolation procedure, could also increase basal glutamate uptake and thus decrease the percentage stimulation by thrombin. Statistical analysis showed that basal uptake levels were not negatively correlated with the percentage stimulation by thrombin. In addition, the mean platelet volume, another parameter indicative of platelet activation, was not correlated with the percentage stimulation induced by thrombin. These results suggest that this variation is patient related.

GS and EAAT2 protein expression

The antibodies against EAAT2 and GS gave rise to single immunoreactive bands at ~79 kDa and ~45 kDa, (fig. 4a). Quantitative analysis (fig. 4b) of the EAAT2 band revealed no difference between ALS patients (2087 +/- 322 arbitrary units (au, n=12) and healthy donors (1812 +/- 242 au (n=14; p=0.494)). However, GS immunoreactivity (45 kDa band) was significantly increased in platelets from ALS patients compared to healthy donors (2347 +/- 164 au (ALS, n=13) vs 1712 +/- 112 au (CTR, n=14); p=0.003) by 37%. GS immunoreactivity was positively correlated with vital capacity (VC%) measured at the day of blood collection (p=0.041, r^2=0.35). The GS immunoreactivity of the two ALS patients, who were not yet with riluzole at the time of blood collection, was in the same range as that of the other patients and exclusion of these patients in the statistical analysis did not change the results.

Discussion

One of the mechanisms implicated in motor neuron death in the pathogenesis of ALS is glutamate excitotoxicity.[23] Evidence for this hypothesis is found e.g. in the studies of *post-mortem* brain tissue, in which glutamate uptake and transporter protein EAAT2 is severely reduced[40-42] and in the fact that riluzole, the only available drug slowing the course of the disease, inhibits glutamate release[198] and increases glutamate uptake at low concentrations.[199,200] Changes in blood parameters of ALS patients may provide valuable tools for early diagnosis, treatment and for mechanistic studies. Therefore we compared GS expression, EAAT2 expression and glutamate uptake in platelets of ALS patients with that in platelets of healthy donors. We found a significant increase in GS expression in platelets of ALS patients, but normal glutamate uptake in resting and activated platelets and normal platelet EAAT2 expression compared to healthy donors.

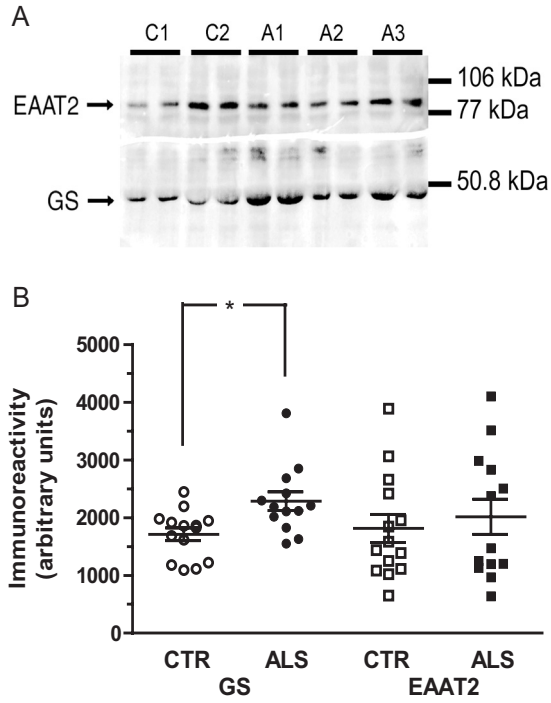

Figure 4. *Western blot analysis of EAAT2 and GS expression in blood platelets. (A) Blot was cut in half and incubated with antibodies against EAAT2 (upper half) and GS (lower half). A single immunoreactive band for EAAT2 is seen at ~79 kDa and for GS at ~45 kDa. C1-2 are platelets lysates from 2 healthy controls and A1-3 from 3 ALS patients. (B) Scatterplot representing individual values of the semi-quantitative analysis of the immunoreactive bands of GS and EAAT2 (* p=0.003, ALS n=12, CTR n=14).The horizontal line indicates the mean ± SEM.*

The finding of an increased GS protein expression in platelets of ALS patients is an indication that alterations in the brain glutamate-glutamine cycle may be paralleled in blood platelets. The increase in GS expression is most likely paralleled by an increase in platelet GS activity. Previous studies in human brain tissue have shown a high degree of correlation between GS expression and GS enzyme activity.[126,127] Our results indicate a positive correlation in ALS patients between GS expression and vital capacity, but this relationship needs to be confirmed in a larger patient group. To date we are not aware of any information on glial GS expression in brain tissue of sporadic ALS patients. Fray *et al*.[201] found no changes in GS activity or protein expression in the upper and lower spinal cord of the G93A SOD-1 mouse model of ALS. However, in several other neurodegenerative diseases, such as Alzheimer's disease[207-209] and temporal lobe epilepsy,[126,127] a reduction in glial GS expression has been reported in brain areas showing neuron death. GS expression has been reported to increase after ischemia *in vivo*[210] after hypoxia in culture[211], and during hyperammonemia.[212,213]
The precise role of GS in blood platelets is unknown. It has been reported that platelets use glutamine in their energy metabolism and that the rate of glutamine utilization is increased in thrombin-activated platelets.[195] The increased GS expression in platelets of ALS patients could reflect an increased glutamate metabolism required to clear glutamate from the blood. It has been suggested that extracellular glutamate in the brain is not only removed via the glial glutamate transporters, but also via brain-to-blood efflux.[214-216] An increased glutamate to glutamine conversion may contribute to the efficiency of this system. However, data on blood and CSF levels of glutamate in ALS are conflicting. Results range from no changes in glutamate levels in plasma and CSF[173,174], to increased levels in plasma[175] or CSF,[37] to normal levels in basal plasma but increased levels after oral glutamate loading.[176] Another function of

platelet glutamate uptake and its subsequent conversion to glutamine could be neutralization of ammonia. Ammonia is toxic to neurons and can freely pass the blood-brain barrier.[217] To our knowledge information on blood or brain levels of ammonia in ALS patients is not available.

Glutamate uptake in resting and thrombin-activated blood platelets was normal in ALS patients. Glutamate uptake was not correlated with age, therefore the difference in mean age between the healthy donors and ALS patients seems not a parameter influencing the results. In line with earlier results[202] activation of platelets with 0.5U thrombin stimulated glutamate uptake in platelets from healthy controls. From our previous studies, we concluded that this thrombin-induced increase in glutamate uptake is mediated by EAAT2 and probably caused by recruitment of EAAT2 from translocating α-granules[202]. Interestingly, also this thrombin-stimulated glutamate uptake did not differ between platelets from ALS patients and controls. In agreement with our glutamate uptake results, we found no difference in EAAT2 expression. In contrast to Zoia *et al.*,[183] we were unable to detect EAAT1 and EAAT3 protein in our platelet homogenates[202]. Although we cannot rule out that platelets express low concentrations of EAAT1 and/or EAAT3, it is unlikely that they make a major contribution to glutamate uptake. Our result suggest that the reduction in glutamate uptake and EAAT2 expression found in the motor cortex of patients with ALS[41,42] is not paralleled by similar changes in blood platelets.

Our observation that platelet glutamate uptake does not differ between ALS patients and controls, is at variance with the results of Ferrarese *et al.*,[177] reporting a reduction in uptake in ALS patients. Since the patient populations used in both studies appear to be rather similar, the most likely explanation for this discrepancy seems to be the differences in methodology used. In fact, we used freshly prepared platelets in all studies, whereas Ferrarese *et al.*[177] determined glutamate uptake after platelet storage in sucrose/DMSO at -80°C until use. A freeze-thaw cycle might cause platelet damage, induce shape changes and can partially activate platelets causing an increase in glutamate uptake. This could explain why Ferrarese *et al.*[177] report high values for K_m (26 ± 13 μM), and V_{max} (178 ± 94 pmol/mg protein /30 min), compared to our data (K_m 3.5 ± 0.9 μM, V_{max} for comparison recalculated in 2.89 ± 0.20 pmol/mg protein/30 min[202]) and those of Mangano and Schwartz[157] (K_m 3.1 ± 0.9 μM, V_{max} 14 ± 6 pmol/mg protein/10 min). However, even in thrombin-activated platelets we did not find any difference in platelet glutamate uptake between ALS patients and healthy volunteers.

In this study we provide evidence for an increase in GS expression in blood platelets of ALS patients. This is another indication of an affected glutamate-glutamine cycle in ALS. Here it was measured during the course of the disease, rather than in an end-stage. The mechanism that induces this increase in GS platelet expression remains unknown, but it is worthwhile to further investigate this phenomenon in relation to the severity of the disease, glutamate clearance from the brain and ammonia metabolism. Moreover, the increase in platelet GS expression may provide a blood marker for ALS and for the effectiveness of therapy.

Acknowledgements

We thank Dr. G.J. Groeneveld for providing the blood samples from the ALS patients. This study was supported by the Epilepsy Fund of the Netherlands, grant no. 01-05 (IWMB) and grant no. A98-16 (GH) as well as by grants of the Dutch Brain Foundation to GH (H00.03) and IWMB GvW was a fellow of the Catharijne Foundation and was supported by the Dirk Zwager-Assink Foundation.

4

Leukocyte glutamine synthetase: a putative peripheral marker for epilepsy

Ineke W.M Bos, Elly J.F. Vereyken, Koen L.I. van Gassen, Marina de Wit, Marije G. Rensen-de Leeuw, Onno van Nieuwenhuizen, Pierre N.E. de Graan

Submitted for publication

Abstract

Background: Early diagnosis of epilepsy in children is of great clinical importance as epileptic insults may cause neuronal damage and impair development in children. Research indicates that the pathogenic process may even start years before clinical symptoms become manifest. Peripheral markers for epilepsy could provide early diagnostic tools and improve treatment. Alterations in proteins involved in glutamatergic signaling are implicated in the pathogenesis of epilepsy. We investigated whether leukocytes express glutamatergic genes and whether some of these genes are suitable as early marker for epilepsy. *Methods*: We used RT-PCR analysis and western blot analysis to show the expression of glutamine synthetase (GS) and EAAT1, 2 and 3. Expression profiling by oligonucleotide microarray analysis was used to compare expression of genes involved in glutamatergic signaling in leukocytes of epileptic children with gender and age-matched controls. Microarray data were confirmed by quantitative PCR (qPCR). *Results*: Leukocyte EAAT1-3 mRNA levels were low while EAAT proteins were not detectable. GS mRNA, protein and enzyme activity was present in human leukocytes. Of the 52 glutamatergic genes present on the microarray, 40 genes were expressed in leukocytes but most of them at relatively low levels. Glutamine synthetase (GS) mRNA is abundantly expressed in leukocytes and its expression was decreased in children newly diagnosed with epilepsy when compared to controls. These microarray data were confirmed by qPCR and further showed that leukocyte GS expression was not changed in the same patients 1.5 years after treatment. Microarray analysis also revealed that GTRAP3-18, a protein associated with EAAT3, is abundantly present on human leukocytes. QPCR analysis showed GTRAP3-18 expression in newly diagnosed epilepsy patients to be as in controls, but increased by 25% after 1.5 years of treatment. *Conclusion*: Leukocytes express genes implicated in glutamatergic signaling and can be used to find peripheral markers for neurological diseases. Of all genes implicated in glutamatergic transmission studied, only GS expression was significantly reduced in children newly diagnosed with epilepsy. Treatment with AEDs does not restore patient leukocyte GS expression, whereas it appears to increase GTRAP3-18 expression. Our data indicate that GS might be a putative early peripheral marker for epilepsy and a target for treatment of epilepsy.

Introduction

Peripheral markers for neurological diseases such as epilepsy can provide early tools for diagnosis. Early diagnosis of epilepsy is of great importance as research indicates that the pathogenic process may start years before clinical symptoms become manifest. Blood markers have been investigated for a number of neurodegenerative diseases. For example, Alzheimer's patients exhibit isoform changes in β-amyloid precursor protein in their blood platelets and this change correlates with the severity of the disease.[138] Recently is has been proposed that unique leukocyte expression patterns may be linked to particular clinical neurological diseases,[218] such as epilepsy, neurofibromatosis and Tourette syndrome. Blood gene expression analysis may provide valuable information about patient pharmacotherapeutic profiles and effectiveness of drug treatment.[218,219]

Alterations in glutamatergic signaling leading to increased excitation and excitotoxicity have been implicated in human epilepsy. These include sustained high levels of glutamate after seizures,[118,120] changes in the expression levels of ionotropic[220-227] and metabotropic[228,229] glutamate receptors and alterations in the expression of splice variants and mRNA editing of several glutamate receptors.[220,230-234] More recently, a decrease in the rate of the brain glutamate/glutamine cycle has been detected using *in vivo* magnetic resonance spectroscopy.[120] This cycle, which involves a tight interaction between neurons and glia, is responsible for the rapid clearance of glutamate from the synaptic cleft. Glutamate is taken up by glial cells, predominantly via excitatory amino acid transporter 2 (EAAT2)[3,11] and converted into glutamine by the glial enzyme glutamine synthetase (GS; EC 6.3.1.2). The non-toxic glutamine is subsequently transported back into neurons, which can reconvert it to glutamate. Indeed, impairments in various components of this cycle, such as glial glutamate transporters[124,125] and glutamine synthetase[126,127] have been found in the hippocampus of patients with temporal lobe epilepsy.

Little is know about the expression of components of glutamatergic signaling cascades in leukocytes. Pharmacological studies established the presence of glutamate receptors on different types of white blood cells.[235,236] RT-PCR analysis and western blotting revealed the presence of mGluR 1, 2/3 and 5 on thymocytes (young T-lymphocytes) and thymic stromal cells in mice[237] and the presence of AMPA receptor subunit GluR3 on human T cells and leukemia cells was confirmed by RT- PCR and western blot.[238] Glutamate transporters EAAT1-3 were reported to be expressed on human lympho-monocytes[183] and macrophages.[239]

The aim of this study is to analyze leukocytes for the expression of genes involved in glutamatergic signaling by RT-PCR, microarray analysis, q-PCR and western blotting in order to identify peripheral markers for epilepsy. The expression profile of genes implicated in glutamatergic signaling in leukocytes isolated from children with epilepsy was compared with age-matched healthy controls, directly after the diagnosis of epilepsy and re-examined after 1.5 years of treatment. Only one of the genes implicated in glutamatergic signaling (glutamine synthetase) was found to be down regulated. Using qPCR we found an increase in GTRAP3-18 expression comparing the same patients before and after treatment. GTRAP3-18 is a protein associated with and modulating the neuronal glutamate transporter EAAT3.[240]

Materials and Methods

Human subjects

Included in this study were six children with epilepsy and six healthy children (control subjects). Control subjects were pair-wise gender- and age-matched to the epilepsy patients. A detailed description of patients and controls is listed in table 1. No general infection was present in any of the patients or controls. The mean age of the epilepsy patients was 5.6 years (range 4-10 years) and the group consisted of 2 boys and 4 girls. Children were referred to our hospital before any medication was taken and a child-neurologist confirmed the diagnosis of epilepsy. The control group consisted of 2 boys and 4 girls with a mean age of 5.8 years (range 4-10 years), who were referred for minor surgery (table 1). To minimize stress for the control children, blood was collected just after induction of anesthesia. Children in the control group were without neurological history.
Blood for RT-PCR, western blot and GS activity analysis was obtained from adult healthy volunteers. Human brain tissue was obtained at autopsy.
Informed consent was provided by all patients or their guardians for the use of tissue for research purposes. The study was approved by the medical ethics review board of the University Medical Center in Utrecht.

Isolation of leukocytes

Human leukocytes were isolated from blood by erythrocyte lysis. Blood was collected by venipuncture in an EDTA vacutainer. Briefly, blood was mixed with 2 volumes of NH_4Cl lysis-solution (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA), kept on ice until lysis was complete and centrifuged at 1200xg for 5 minutes at 4ºC. Leukocytes were washed twice, first with NH_4Cl lysis-solution and then with PBS and were resuspended in 50 µl PBS. Leukocytes were lysed in 1 ml TRIzol (Gibco Brl, Life Technologies) and stored at -80°C until RNA isolation. For western blot and GS activity assay, part of the leukocyte preparation from adult blood donors, was diluted in lysis buffer (100 mM Tris-HCl, 1mM EGTA, 1mM EDTA, supplemented with protease inhibitors (Complete©, Roche Diagnostics)) and stored at -80ºC until use.

RT-PCR analysis

Total RNA was extracted following the TRIzol isolation method according to the manufacturer's protocol. mRNA was reverse-transcribed using 200 U superscript II (Invitrogen, Life Technologies) and oligo dT primer (Invitrogen) in a Peltier Thermal Cycler (MJ research) according to the manufacturer's protocol. cDNA products were amplified using specific primers listed in table 2. Sense and antisense primers (Isogen Bioscience BV) were designed to span an exon-exon junction to discriminate between genomic DNA and mRNA templates. Hot-start PCR was performed twice (2x 30 cycles) for EAAT1-3 and once for GS. The identity of the products was verified by amplicon size and sequencing; products were extracted from a 1% agarose gel using the PCR-extraction kit (Qiagen). Purified PCR products were then sequenced using

Table 1. Summary of clinical data of patient and controls

Epilepsy	Age (y,m)	Gender	Diagnose	AED treatment	Clinical outcome after 1.5 years
1	4,5	M	Rolandic epilepsy	-	seizure free
2	8,2	F	idiopathic generalized epilepsy	VPA (disc), LTG + LEV	recurrent seizures
3	4,11	F	idiopathic generalized epilepsy	VPA	lost for follow up
4	5,8	M	C.P.S., sec. gen.	VPA	seizure free
5	7,0	F	recurrent FS (>10)	VPA	seizure free
6	3,8	F	CAE	VPA (disc), ESM	seizure free
Control	Age (y,m)	Gender	Diagnose		
1	5,11	M	urology surgery		
4	8,5	F	urologic surgery		
5	4,2	M	strabismus surgery		
9	7,4	F	ENT surgery		
10	4,3	F	urologic surgery		
12	5,1	F	ENT surgery		

CAE = childhood absence epilepsy; CPS =complex partial seizures, secondarily generalized; disc = discontinued; ENT = ear-nose-throat; ESM = ethosuximide; FS = febrile seizures; LEV = levetiracetam; LTG = lamotrigine; m = months; VPA = valproate; y=years

the CEQ Dye Terminal Cycle Sequence (DTCS) Quick Start Kit and a CEQ 2000 Beckman Coulter sequencer according to the manufacturer's instructions.

Western blot

Primary antibodies used for western blot analysis were polyclonal guinea pig-anti-EAAT1 1:5000 (Chemicon International), polyclonal guinea pig-anti-EAAT2 1:2000 (Chemicon International), polyclonal rabbit-anti-EAAT3 1:500 (Alpha Diagnostics Inc.) and monoclonal mouse-anti-GS 0.25µg/ml (Transduction Laboratories).
Proteins in human leukocyte (5 or 40 µg) and rat (0.5 or 5 µg) or human (5 µg) cortex homogenates, used as positive controle, were separated by 10% SDS-PAGE and transferred by semi-dry electroblotting for 1.1 hr at 400 mA onto polyvinylidene difluoride membranes (PVDF, Amersham). Membranes were blocked for 1 hr at RT with 5% lowfat dry milk in 50mM Tris-buffered saline containing 0.1% Tween-20 (TBSt) and BSA (10% for anti-EAAT1 and EAAT2, 5% for EAAT3 and 1% for anti-GS). Membranes were incubated with primary antibody overnight at 4°C in TBSt (plus 10% BSA for anti-EAAT1 and 5% BSA for EAAT3), washed and incubated with alkalyne phosphatase conjugated secondary antibody for 1 hr at RT (1:2000 goat-anti-guinea pig IgG, 10% BSA in TBSt or 1:2000 goat-anti-rabbit IgG or 1:5000 goat-anti-mouse IgG in TBSt). Subsequently membranes were washed and incubated with enhanced chemofluorescence substrate (ECF, Amersham) and visualized using a Fluor-S MultiImager (BioRad).

Microarray analysis

Microarray analysis was performed as described by Roepman *et al*. (2005).[241] Briefly, total RNA was isolated from freshly isolated leukocytes using TRIzol reagent, purified by DNase treatment (DNAse kit, Ambion) and by the RNeasy MinElute Cleanup kit (Qiagen). RNA quality was analyzed using the 2100 Bioanalyzer (Agilent Technologies Inc). cDNA was synthesized using T7 oligo(dT)24VN primer (Ambion) and Superscript II reversed transcriptase (Invitrogen). cDNA was *in vitro* transcribed using the T7 Megascript kit (Ambion) and cRNA quality was analyzed using the 2100 Bioanalyzer. Cy3 or Cy5 fluorophores (Amersham) were coupled to 1500 ng cRNA of both control and patient leukocytes.

Fragmented (RNA Fragmentation Reagents, Ambion) Cy3 labeled control leukocyte cRNA (1000ng) and 1000 ng of fragmented Cy5 labeled patient leukocyte cRNA were pooled and hybridized overnight at 42˚C in a water bath. All patients and controls were hybridized twice on human oligonucleotide arrays,[241] switching the fluorophores to minimize dye specific effects. Slides were washed manually and immediately scanned in the G2565AA DNA Microarray Scanner (Agilent Technologies Inc.). Scanned slides were quantified and background corrected with Imagene (version 5.6.1) and Lowess normalized as described by van Bakel et al, 2005.[242] Gene expression was normalized per chip to the 50th percentile, visualized in Genespring (version 7.2, Biodiscovery Inc.) and statistical analysis was performed by Statistical Analysis of Microarrays (SAM[243]). Only genes related to glutamatergic neurotransmission were analyzed.

qPCR

For quantitative PCR (qPCR) cDNA synthesis was preceded by DNase treatment (DNaseI, Invitrogen) according to the manufacturer's protocol. RT-PCR was carried out using Superscript II (Invitrogen) and oligo-dT primers according to the manufacturer's protocol, without DTT and RNA-guard.

The qPCR reaction was performed using the LightCycler (Roche) and the Fast Start SYBRgreen master mix (Roche) according to the manufacturer's protocol. Reactions were performed in a total volume of 10 µl containing 2 µl cDNA, 2-5 mM $MgCl_2$, 0.5 µM of each primer and 1 µl of the SYBR Green reaction mix. Cycling conditions were as follows: initial denaturation/FastStart Taq DNA polymerase activation at 95°C/10 min, 40 cycles of denaturation at 95°C/10 sec, annealing at primer-pair specific annealing temperature and time (55°-59°; 5-11 sec) (with fluorescence acquisition at the end of each annealing stage) and elongation at 72°C/9 sec (table 3). Specificity was verified by melting temperature determination at the end of each run and on an agarose gel.

Primers were designed for qPCR analysis with specifications recommended in the LightCycler manual (Isogen Biosciences BV, HPLC purified). The PCR reaction was optimized for each primer pair for annealing temperature and $MgCl_2$ concentration.

PCR efficiencies were determined for each gene by serial dilution series of cDNA. Primer specifications are given in table 3. Cycles of threshold (Ct) for each sample were determined using the second derivative maximum (Light Cycler Software, version 3.5, Roche). Gene expression was calculated as normalized ratio (Rn), which

is a measure for the amount of cDNA present in a sample relative to a calibrator sample measured in the same qPCR experiment and normalized to a reference gene. A pool of leukocyte cDNAs from different donors was used as calibrator. Succinate dehydrogenase subunit A (SDHA),[244] β-Actin (ACTB) and cyclophilin A (PPIA)[245] were used as reference genes. All samples were analyzed in duplicate and reported as mean.

Data were analyzed using paired Student's t-test. P-values <0.05 were considered significant.

Table 2. Overview of RT-PCR primers

Gene	Accession no	Primer sequence 5'- 3'	Product (bp)	Ann.T (ºC)
EAAT1	gi:8118688	f:cgagctgtggtctattacatg r:gcttgcagcaaccctccaat	700	52
EAAT2	gi:498256	f:aatggatcctttcgttgcctggaaga r:gacaagcttatttctcacgtttccaag	620	54
EAAT3	gi:66773029	f:gctcattgctgtcgactggctc r:gtgaggtctgggtgaatgagatgg	260	62
GS	gi:22749654	f:agaccaatttgaggcacacc r:cataggctctgtctgctccc	530	62

RT-PCR primers (F: forward, R: reverse), Genbank accession numbers of sequences used as template, product lengths (bp:base pairs) and annealing temperatures (Ann.T).

Table 3. Overview of qPCR primers.

Gene	Accession no	Primer sequence 5'- 3'	Product (bp)	$MgCl_2$ (mM)	Ann.T / time	E
GS	gi:22749654	f:acttcgcaagcggcacca r:ttggctacaccagcagaaaa	125	5	55ºC / 5	2.13
GTRAP 3-18	gi:37588901	f: gatttccattgtggggttt r:aggttcctaagtctcaacga	270	4	57ºC / 11	1.98
B-Actin	gi:15426604	f:tgctatccctgtacgcctct r:taatgtcacgcacgatttcc	220	5	59ºC / 9	2.05
Cyclophilin	gi:33869826	f:ctgagcactggagagaaagga r:gcagatgaaaaactgggaac	230	5	59ºC / 9	2.06
SDCA	gi:62087561	f:tgtggagtattttgccttgga r:ttgctcttatgcgatggatg	100	5	55ºC / 5	2.14

qPCR primers (f: forward primer, r: reverse primer), accession number: Genbank accession numbers of sequences used as template, product length (bp=base pairs), annealing temperature (Ann T) and cycle time (Ann. time), optimal MgCl2

GS enzyme activity assay

GS activity was determined according to Levintow (1954) in human leukocytes (3 and 6 µg/µl) and hippocampus (2 µg/µl) homogenates. Briefly, GS-activity was measured in duplicate over a 60-120 minute incubation at 37°C by mixing 20 µl homogenate with 80 µl reaction mixture (200 mM L-glutamine, 0.16 mM ADP, 25 mM NH_2OH, 25 mM Na_2AsO_4, 50 mM Imidazole, 1.5 mM $MnCl_2$). The reaction was stopped by adding 200 µl stop solution (89 mM $FeCl_3$, 0.6 M HCl, 0.5 M $HClO_4$). Glutamyl-τ-hydroxamate ferric chloride complex formation was measured spectophotometrically at 490 nm, after clearing the reaction mixture by centrifugation. Absorption was corrected for background in each sample. The amount of GS enzyme activity in the samples was determined using a standard curve with predetermined amounts of glutamyl-τ-hydroxamate in the homogenization buffer. GS activity was expressed as nmol/min/mg protein. One freeze-thaw cycle did not affect GS activity.

Results

Expression of EAAT1, 2, 3 and GS in human leukocytes

The presence of several transcripts of the glutamatergic system in human leukocytes was determined using RT-PCR analysis. After isolation of total RNA, cDNA was generated with oligo dT primers. PCRs were conducted with various specific primer sets for EAAT1-3 and GS (table 2). For EAAT1, the first PCR revealed no product, a second PCR of 30 cycles with the same primers set and using the first PCR products as template, generated a band at 700 bp as expected for EAAT1 (fig 1). After the second PCR for EAAT2 in human leukocytes, a PCR product of the expected size (620 bp; fig 1) was present. With the same method, human leukocytes showed mRNA for EAAT3 (260 bp) (fig 1). A single PCR for GS revealed a band of the appropriate size (530 bp), indicating that GS mRNA is more abundant than EAATs in human leukocytes. The identity of all amplified products was confirmed by sequencing.
Consistent with the lower expression levels of EAAT1, EAAT2 and EAAT3 mRNA, we were unable to detect these proteins in leukocyte homogenates on western blots (fig 2), even though high protein levels were loaded. In rat cortex controls we readily detected immunoreactive bands at the appropriate molecular weight for EAAT1, EAAT2 and EAAT3 (fig 2). In leukocytes homogenates we detected a single GS immunoreactive band at 40 kDa, migrating at the same position as in human cortex homogenate (fig 2). Enzyme activity was 4.84 ± 0.25 nmol/min/mg protein in human leukocytes and 82.83 ± 8.2 human hippocampus homogenates (mean ± SEM). Enzyme activity was protein and incubation time dependent.

Expression of genes implicated in glutamatergic signalling in leukocytes of children newly diagnosed with epilepsy.

As RT-PCR provided evidence for the presence of transcripts coding for proteins

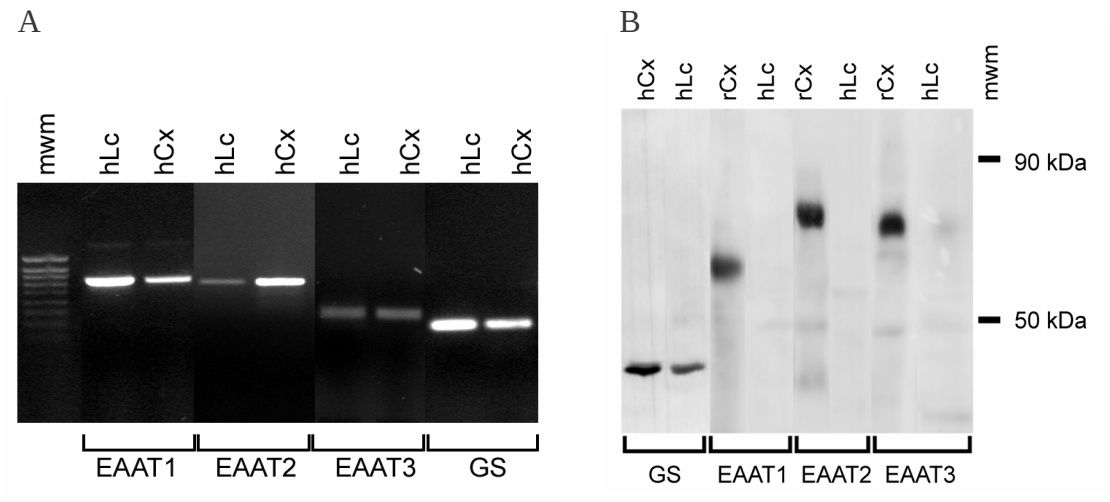

Figure 1. *mRNA and protein expression of EAAT1, EAAT2, EAAT3 and GS in human leukocytes.* ***A****: RT-PCR analysis revealed that human leukocytes (hLc) and human cortex (hCx) express mRNA of EAAT1, 2, 3 and GS. mwm = 100 bp molecular weight marker.* ***B****: Western blot analysis revealed GS protein immunoreactivity in homogenates of human leukocytes. EAAT1, EAAT2 and EAAT3 proteins were not detectable in human leukocytes. In all experiments human or rat cortex (rCx) were used as positive control. Omission of the primary antibody abolished all immunoreactive staining (data not shown).*

involved in glutamate neurotransmission, expression profiling with microarrays was applied to compare leukocytes of epilepsy patients with healthy controls. Leukocytes were found to express a large number of genes involved in glutamatergic signaling, including genes coding for ionotropic and metabotropic glutamate receptors and genes related to the glutamate-glutamine cycle. In total 52 genes implicated in glutamatergic signaling were selected for analysis (table 4). Most of these genes were expressed at relatively low levels in leukocytes. Six genes were highly expressed (signal above 1000) and 12 genes were considered absent, as their expression was below 2 times background values (signal below 100). Statistical analysis (SAM: $q < 0.001$), on all the genes present on the microarray, revealed 11 genes to be up-regulated and 779 genes down-regulated in newly diagnosed children with epilepsy compared to age- and sex-matched controls. From these 779 down-regulated genes only GS has been implicated in glutamatergic signaling. Normalized GS gene expression (table 4) in this microarray study was 0.461, indicating a GS transcript reduction of at least 50%. Normalization of GS expression against the house keeping genes used in the qPCR analysis gave similar results. The microarray results of EAAT1, EAAT2, EAAT3, GS and GTRAP3-18 are depicted in figure 2.

GS mRNA expression in leukocytes of children with epilepsy.

Changes in GS expression levels in leukocytes of epilepsy patients were further analyzed by qPCR analysis. Relative expression of GS was compared with three housekeeping genes (SDHA, cyclophilin A, β-actin). No differences were detected in expression levels of these housekeeping genes between the two groups.
Confirming microarray results, GS expression in leukocytes of epileptic children was significantly lower than in healthy controls. Normalized ratios (Rn) of GS versus

Table 4. Expression levels of glutamate related genes.

Gene name	Ref Seq	Ratio Epilepsy vs. Control	Relative expression
Ionotropic glutamate receptor subunits			
NMDA receptor subunits			
GRIN1	NM_000832	1.031	144
GRIN2A	NM_000833	1.049	93
GRIN2B	NM_000834	1.081	93
GRIN2C	NM_000835	0.915	628
GRIN2D	NM_000836	1.074	296
GRIN3A	NM_133445	1.001	291
GRIN3B	AC004528	1.017	145
GRINL1A	NM_015532	1.141	276
AMPA receptor subunit			
GRIA1	NM_000827	1.014	210
GRIA2	NM_000826	0.997	113
GRIA3	NM_000828	1.025	526
GRIA4	NM_000829	1.407	30
Kainate receptor subunits			
GRIK1	NM_000830	1.007	687
GRIK2	NM_021956	1.118	166
GRIK3	NM_000831	0.873	31
GRIK4	NM_014619	1.034	74
GRIK5	AJ249209	0.968	132
Glutamate delta receptors			
GRID1	AB033046	1.058	122
GRID2	NM_001510	1.025	99
Glutamate receptor interacting protein			
GRIP1	AJ133439	1.034	167
Metabotropic glutamate receptors			
GRM1	NM_000838	0.983	74
GRM2	NM_000839	0.983	338
GRM3	NM_000840	1.129	81
GRM4	NM_000841	0.892	630
GRM5	NM_000842	0.970	75
GRM6	NM_000843	1.043	244
GRM7	NM_000844	0.978	69
GRM8	NM_000845	1.061	186
Glutamate transporters			
S83374	S83374	1.058	151
SLC1A1	NM_004170	0.936	251
SLC1A2	NM_004171	0.993	66
SLC1A3	NM_004172	0.982	442
SLC1A4	NM_003038	0.929	114
SLC1A6	NM_005071	1.069	445
SLC1A7	NM_006671	1.014	1225
SLC25A22	NM_024698	1.116	532

Table 4 continued

Gene name	Ref Seq	Ratio Epilepsy vs. Control	Relative expression
Glutamate transporter associated protein			
ARL6IP5	NM_006407	1.003	1461
Enzymes			
GAD1	NM_013445	1.127	164
GAD2	NM_000818	1.026	135
GCLC	NM_001498	1.048	932
GCLM	NM_002061	1.035	5377
GLS	NM_014905	0.951	410
GLUD1	NM_005271	0.816	4838
GLUL	NM_002065	** 0.461	3311
GLULD1	NM_016571	0.966	102
TGM1	NM_000359	1.259	85
TGM2	NM_198951	0.975	134
TGM2	NM_198951	1.014	184
TGM3	NM_003245	0.865	310
TGM4	NM_003241	1.046	101
TGM5	NM_004245	0.994	440
TGM7	NM_052995	1.086	24322

Ref Seq = reference sequence accession number, ratio epilepsy vs. control = expression of the genes in leukocytes of newly diagnosed epilepsy patients divided by gender- and age-matched controls (** = q < 0.001), relative expression = average fluorescence intensity.

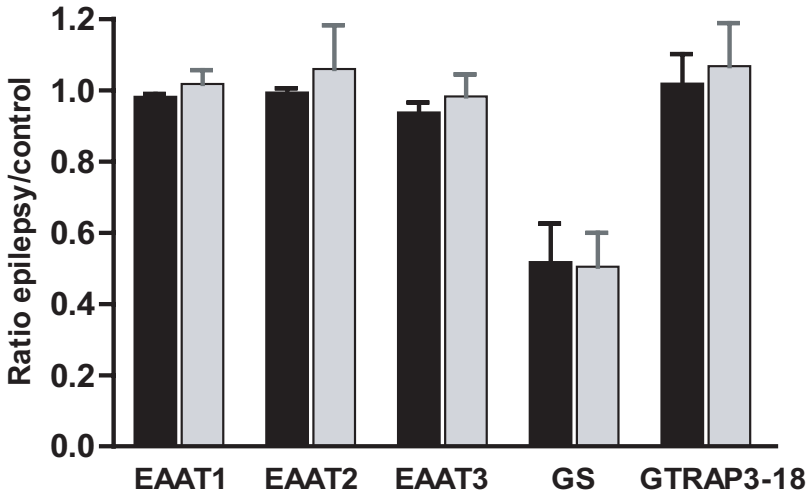

Figure 2. *Microarray analysis of 5 genes related to the glutamate-glutamine cycle. Expressed as the ratio of leukocyte expression level in newly diagnosed patients divided by expression level in control subjects (black bars). This ratio is also expressed using the expression levels normalized to SDHA expression also available on the microarray (grey bars). Data expressed ± SEM, ** q<0.001, SAM analysis, significantly decreased.*

SDHA expression were 0.78 ± 0.10 in controls (n=6) and 0.45 ± 0.14 in epilepsy patients (n=6, Rn ± SEM, p=0.015, fig 3), showing a 45% reduction in GS mRNS expression. Normalization of GS expression against the two other housekeeping genes gave similar results. One patient (no.5) included in this study, who showed the highest GS expression level, was eventually not diagnosed with epilepsy. She suffered from multiple recurrent febrile seizures in the past, but did not show spontaneous seizures. GS expression in blood leukocytes was reanalyzed after 1.5 years treatment. All children, except one (no.1), received AED treatment. One patient (no.3) was lost for follow up. Clinical outcome was good except for patient 2. This child suffered from seizures despite multidrug treatment (table1). The expression level (Rn) of GS versus SDHA expression was 0.37 ± 0.05. This was similar to the levels measured just after diagnosis, showing that after 1.5 years treatment GS expression was not significantly changed (p=0.84, n=5, fig 3).

GTRAP3-18 expression in leukocytes of epilepsy patients

From the microarray analysis we learned that GTRAP3-18 (a.k.a. ARL6IP5, JWA) mRNA was abundantly expressed in human leukocytes. We determined leukocyte expression levels of this gene with qPCR in newly diagnosed epilepsy patient compared to controls and reanalyzed patient leukocyte expression of GTRAP3-18 after 1.5 years. QPCR showed that leukocyte GTRAP3-18 mRNA expression normalized to SDHA mRNA expression was not different in newly diagnosed epilepsy patients compared to age- and gender-matched controls (1.12 ± 0.11 (epilepsy, n=6) vs 1.27 ± 0.08 (controls, n=6, Rn ± SEM, fig 3). Reanalysis after 1.5 years treatment revealed a small but significant increase (+25%) in leukocyte GTRAP3-18 expression normalized by SDHA expression (1.59 ± 0.14, n=5, p=0.0302).

Discussion

The current study used microarrays to identify expression of genes implicated in glutamatergic signaling in human leukocytes. In total almost 880 genes on the array were differentially expressed in leukocytes of newly diagnosed children with epilepsy. Human leukocytes express 40 of 52 genes on the array implicated in glutamatergic signaling. Of these 40 genes only glutamine synthetase (GS) mRNA was differentially expressed (45% reduction). Using qPCR we confirmed that indeed leukocyte GS mRNA is reduced by about 45% in newly diagnosed children with epilepsy compared to controls. Detailed re-analysis of the microarray data using the same house keeping genes as in the qPCR gave similar results. This shows in an independent manner that the expression of these genes remains unaltered and it also emphasizes the reliability of the microarray data. One child with epilepsy expressed relative high levels of GS mRNA as measured by qPCR and microarray analysis. Re-evaluation of the clinical history of this patient revealed that this patient suffered from repetitive complex febrile convulsions, but was not diagnosed with epilepsy. Since this was discovered

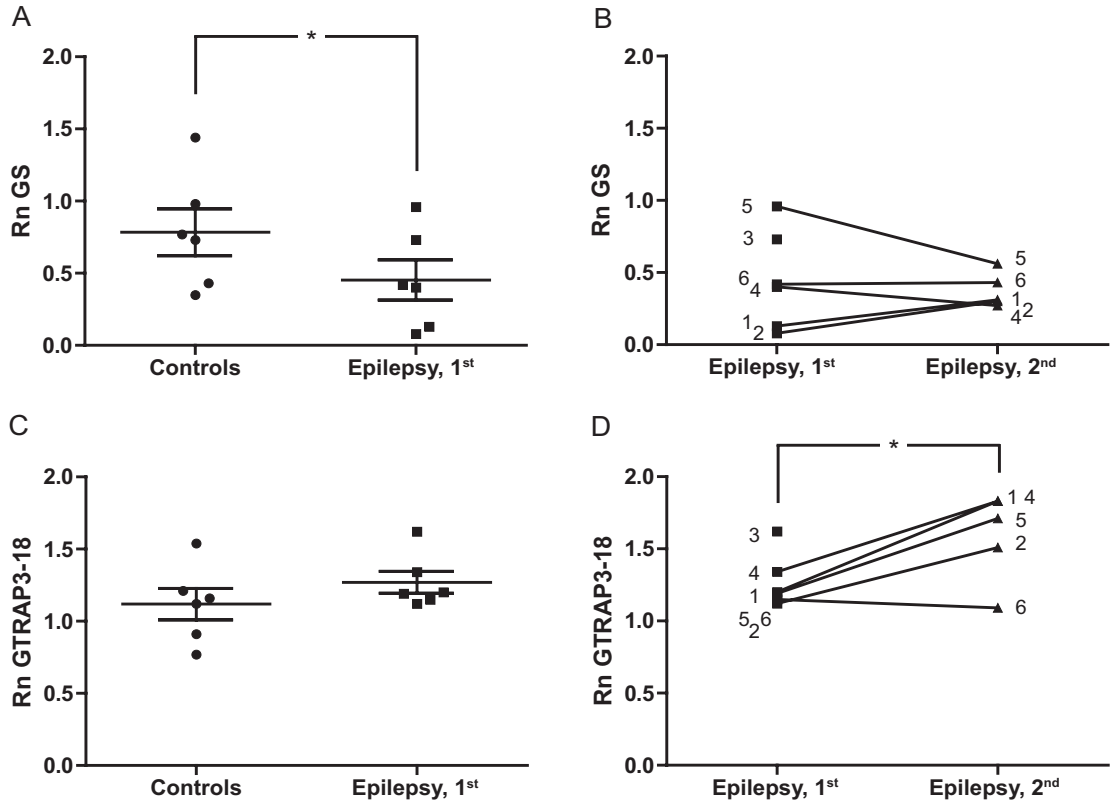

Figure 3. *Quantitative PCR for GS and GTRAP3-18 mRNA in leukocytes of epileptic children. Normalized ratio (Rn) ± SEM of GS (A) and GTRAP3-18 (C) in leukocytes of epileptic children relative to the reference gene SDCA. Rn GS is decreased compared to healthy controls (p=0.0185) and GTRAP is not changed. One patient (no.5) included in this study, who showed the highest GS expression level, was eventually not diagnosed with epilepsy. She suffered from multiple recurrent febrile seizures in the past, but did not show spontaneous seizures.*
Normalized ratio (Rn) ± SEM of GS mRNA (B) and GTRAP3-18 mRNA (D) in epileptic children at diagnosis and after 1.5 years. GS expression is unaltered compared to the first measurement, but GTRAP3-18 is increased (p=0.0302). Patient numbers correspond with table 1. Controls n=6, epilepsy 1st (newly diagnosed) n=6, epilepsy 2nd (after 1.5 years) n=5.

after the analysis we have included this patient in the analysis. Leukocyte GS mRNA expression in the same patients was re-analyzed with qPCR 1.5 years later. GS expression was not significantly different from the first measurement and thus still decreased. All but one patient were using AEDs at that time. The initial treatment was valproate, although two patients discontinued and were using different AEDs at the time of second blood collection. Four patients were seizure free, but one patient still suffered from recurrent seizures despite multidrug treatment. Thus, these preliminary results indicate that GS mRNA expression remains low despite the treatment with antiepileptic drugs and/or absence of seizures.
Our data indicate a reduction of GS activity in leukocyte of children with epilepsy, assuming that the changes in mRNA are translated into GS activity. In brain tissue at least a close correlation has been found between GS protein and enzyme activity

levels.[126,127] In this study, we demonstrated GS protein enzyme activity in human leukocytes. Due to the small seize of the blood sample from the children and the low leukocyte GS activity we were unable to directly correlate expression levels and functional GS. The decreased level of GS expression in leukocytes may reflect a decrease in GS expression in the brain, where it is very important in astrocytes for reducing glutamate levels, due to its uptake from the exterior by their glutamate transporters. Impairment of this function can result in hyperexcitability.[246] Reduction of GS expression has been reported in the hippocampus of TLE patients with hippocampal sclerosis.[126,127] Our study indicates that impaired GS expression may have a genetic background and thus may be causal in epilepsy. For instance if regulatory domains of this gene harbor mutations, expression might be influenced, paralleling our findings. Complete knock-down of one allele is also a possibility, as we found a 50% reduction in transcript level. No linkage has been found in the chromosome region of GS (1q31), suggesting it does not play a role in Mendelian forms of epilepsy. For this gene no association studies have been performed in sporadic epilepsy patients (for example temporal lobe epilepsy). So, whether this gene is involved in polygenic (e.g. multifactoral) types of epilepsy remains to be answered. Another explanation for the reduced GS expression in leukocytes could be some kind of signaling from the brain to the blood, adapting to the epileptic state. This however, seems less likely because of the large effect (50% reduction) and a compensatory increase rather than a decrease in GS activity would be expected.

We also determined the leukocyte expression of GTRAP3-18, despite the fact that the microarray analysis did not show a difference in the leukocytes of newly diagnosed epilepsy patients. This gene is also abundantly present in leukocytes and other peripheral tissues[247] and is known under several different names e.g. ARL6IP5 (ADP ribosylation-like factor 6 interacting protein 5), JWA and others. This protein interacts with the C-terminal of EAAT3 and reduces glutamate transport by lowering substrate affinity.[240] QPCR analysis of leukocyte GTRAP3-18 expression in newly diagnosed patients showed no difference in expression level. However, re-analyzing the same patients after 1.5 years treatment revealed a small but significantly increased level of GTRAP3-18. Our data indicate that AED treatment may increase GTRAP3-18 expression, thus down-regulating EAAT3-mediated glutamate uptake. As controls are 1.5 years younger than the epilepsy patients after 1.5 years, an age effect can not be excluded. The group of patients is very small and further experiments need to be performed to investigate the potential of GTRAP3-18 expression as marker for effectiveness of pharmacotherapy

In this study we found evidence that leukocyte GS expression is persistently low in epilepsy patients and that leukocyte GTRAP3-18 expression may be increased by AED treatment. Due to the small number of patients we were unable to analyze expression in different types of epilepsies, or after treatment with different AEDs. Moreover, we need to study specificity of the markers by including patients with other neurological diseases and control for the short-term effects of anesthesia or possible surgery-related stress. In addition, it will be very interesting to study GS expression in adult patients with various epilepsy syndromes, including patients operated to resect the epileptic focus.

Little is know about the physiological role of GS and GTRAP3-18 in leukocytes. Several components of the glutamatergic signaling pathways are expressed in leukocytes[183,235-237,239] and some functional effects of glutamate on leukocytes have been described.[238,248] GS might plays a role in energy metabolism.[249,250] GTRAP3-18 in brain, functions as inhibitor of glutamate reuptake by interacting with EAAT3[240,247] and EAAT3 is predominantly expressed on neurons.[5,8] No information is available on the expression of GTRAP3-18 in the human epileptic brain. As leukocyte EAAT3 gene expression is very low, a pathophysiological role of the 25% increase in GTRAP3-18 seems unlikely. Unless of course, GTRAP3-18 serves other yet unknown functions in leukocytes.

Peripheral GS and GTRAP3-18 expression might provide unique markers for syndromes like epilepsy. Whether these expression changes in leukocytes are specific for epilepsy and are not found in other neurological and neurodegenerative diseases remains to be investigated. This study provides the first step towards identifying peripheral markers for epilepsy. Identification and validation of such markers is essential in the early diagnosis of epilepsy and may contribute to choice of drug-therapy in individual patients.

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5

Transient changes in glutamate transporter expression after pilocarpine induced SE in juvenile rats

Ineke W.M Bos, W. Saskia van der Hel, Gineke Wassink, Onno van Nieuwenhuizen, Pierre N.E. de Graan.

Submitted for publication

Abstract

Temporal lobe epilepsy (TLE) is a common epileptic disorder in which seizures arise from the temporal lobe. Excitotoxicity due to malfunction of the glutamate-glutamine cycle is implicated in the pathogenesis of this disease. Excitatory amino acid transporters play a key-role in this cycle. The juvenile lithium-pilocarpine model is a status epilepticus (SE) model in the rat commonly used to study aspects of human TLE. In the present study we investigated longitudinal changes in glutamate transporter expression in the hippocampus after lithium-pilocarpine induced SE. We induced SE at P21 with a moderate dose of pilocarpine (40 mg/kg). At 19 weeks after SE about 45% of the rats showed spontaneous recurrent seizures, but, as expected, no indication was found for neuronal cell death or mossy fiber sprouting in the hippocampus.

GLAST and GLT1 immunoreactivity were increased in the stratum lacunosum-moleculare of the hippocampus and in a particular cell population in the hilus of the dentate gyrus at 2 weeks after pilocarpine induced SE (+SE). At this time-point vimentin immunoreactivity was increased in the same regions, but glutamine synthetase immunoreactivity, another glial protein active in the glutamate-glutamine cycle, was unchanged. Changes found in +SE rats 2 weeks after SE were transient, as they were not found in rats 4, 8 or 19 weeks after SE. In some animals that did not reach full SE during the pilocarpine treatment (–SE), these transient changes in GLAST and GLT1 expression were also found, but at 4 weeks after pilocarpine induced SE. No changes were observed in immunoreactivity of the neuronal glutamate transporter EAAC1 at any of the time-points studied.

Our data clearly show transient changes in glial glutamate transporter expression in young rats 2 weeks after pilocarpine-induced SE, which does not result in neuronal cell loss or mossy fiber sprouting. Possibly, these changes reflect temporary changes in glutamatergic transmission and may be part of the complex mechanism leading to epileptogenesis.

Introduction

Epilepsy is one of the most common neurological disorders affecting 1-2 % of the total population. It is a chronic disorder characterized by recurrent seizures. In temporal lobe epilepsy (TLE) the seizures arise from the temporal lobe. Most patients with TLE can be treated with anti-epileptic drugs, but about 20% of the patients are pharmaco-resistant.[67]

Impairments in glutamatergic signaling have been implicated in human epilepsy. For instance, in the human hippocampus analysis by microdialysis showed that the extracellular glutamate concentration is increased just before and during a seizure.[118] Moreover, the rate of glutamate-glutamine cycling measured by magnetic resonance spectroscopy was decreased in the human epileptic hippocampus.[120]

Indeed alterations in the expression of proteins associated with glutamatergic neurotransmission have been identified in human hippocampi, resected during surgery for treatment of intractable TLE. These include changes in the expression of ionotropic[220-223] and metabotropic[228,229] glutamate receptors, as well as in glutamate transporters[124,125] and in the glutamate converting enzyme glutamine synthetase.[126,127]

Synaptically released glutamate is normally taken up by glutamate transporters. This uptake is essential in glutamatergic transmission, as it keeps the extracellular glutamate concentration low, thereby limiting the excitatory signal and preventing excitotoxicity. Five mammalian subtypes of glutamate transporters have been cloned, EAAT1 (a.k.a. GLAST[5,6]), EAAT2 (a.k.a. GLT1[5,7]), EAAT3 (a.k.a. EAAC1[5,8]), EAAT4[9] and EAAT5.[10] These subtypes show high sequence homology, but have a distinct expression pattern in the central nervous system and in peripheral tissues. EAAT1 is mainly located in astrocytes of the cerebellum and moderately in the hippocampus and the superior colliculus.[179,251] EAAT2 is expressed in astrocytes throughout the CNS[179,252] and EAAT3 is neuron specific and specifically enriched in the hippocampus, cerebellum and basal ganglia.[179,253] EAAT4 is located in purkinje cells of the cerebellum[9,254] and EAAT5 is only present in the retina and not elsewhere in the CNS.[10] The glial transporters EAAT1 and EAAT2 are thought to be responsible for more than 90% of the total glutamate transport in the brain.[3] EAAT2 expression was found to be increased in the hippocampus of TLE patients without hippocampal sclerosis.[124] In the hippocampus of patients with hippocampal sclerosis expression of EAAT1 was found to be moderately decreased and EAAT3 expression was increased in surviving neurons.[124]

Post-status epilepticus models in the rat are commonly used in epilepsy research because they mimic certain aspects of human TLE. In these models, it was shown with microdialysis that after pilocarpine and kainate seizures glutamate levels increase both in adult and juvenile animals, regardless of the different nature of the seizure inducing chemicals.[121,122] Pilocarpine had no direct effect on hippocampal glutamate uptake[255] or on synaptosomal glutamate release,[256] indicating that the rise of glutamate level is due to the seizure activity. In the pilocarpine model only EAAC1 (EAAT3) expression has been studied so far. In the adult and the juvenile pilocarpine model EAAC1 mRNA was found to be increased in individual granule cells during the latent phase as well as during the phase in which animals have spontaneous

recurrent seizures (SRS).[257,258]
Although hippocampal tissue, resected during surgery for the treatment of temporal lobe epilepsy is invaluable to investigate molecular mechanisms, it is obtained after the patients have suffered from recurrent seizures for many years. Therefore, it is difficult to distinguish cause and consequence in this tissue. In order to obtain more insight into a possible causal role of EAATs in temporal lobe epilepsy, we performed a longitudinal study on hippocampal EAAT protein and mRNA expression after pilocarpine injection in juvenile (P21) rats. Juvenile rats were used, because their age compares well with the time of onset of human TLE, an age at which the brain may have a different vulnerability to seizures than at adulthood.

Materials and Methods

Animals

Male Wistar rats (Charles River Laboratories, Schulzfeld, Germany) arrived at postnatal day 8 (P8) in litters of 10 pups with a foster mother. Rats were weaned and housed individually at P21. Animals were housed in a temperature and humidity controlled room with tap water and standard rat chow freely available and lights on between 7AM and 7PM. The experimental procedures were approved by the Ethical Committee for Animal Experiments of Utrecht University.

Lithium-Pilocarpine induced SE

At P20, rats were injected with lithium-chloride (Merck, Darmstadt, Germany) 3 mmol/kg intraperitoneally (i.p.) to potentiate the pilocarpine treatment. At P21, 18-20 hrs after lithium treatment, animals were injected with methyl-scopolamine (1 mg/kg , Sigma, St. Louis, MO, USA) i.p. to reduce the peripheral side effects of pilocarpine. Rats were placed in individual plastic cages for behavioral observation. Thirty minutes later, pilocarpine (40 mg/kg, Sigma, St. Louis, MO, USA) was administered subcutaneously. The behavior of the animals was observed and classified in six stages according to Racine (1972[259]). After one hour of SE, rats were injected with diazepam (4 mg/kg i.p., Centrafarm Sevices BV, Etten-Leur, the Netherlands) to suppress seizures and reduce the mortality rate.
Control animals were handled and housed in the same manner. Two groups of control animals were made. A –PC group received full treatment except that pilocarpine injections were replaced by an equal volume of saline. A second control group (SHAM) only received saline injections.

Tissue collection and processing

At 2, 4, 8 or 19 weeks after pilocarpine SE, animals were sacrificed by i.p. injections of 300 mg/kg pentobarbital. Animals were perfused transcardially with 100 ml of saline containing 500 U of heparin (Leo Pharma B.V., Breda, The Netherlands),

followed by 200-250 ml of 4% (w/v) phosphate buffered paraformaldehyde (pH 7.4). Brains were dissected and postfixed overnight at 4˚C, dehydrated and embedded in paraffin.
For Timm-stain and functional studies, animals were decapitated at 19 weeks after pilocarpine treatment. Brains were dissected and the hemispheres were split. One half was immediately frozen and stored at -80˚C until used for Timm-stain, the other half was placed in ice-cold Na^+-HepesTris buffer (see section glutamate uptake) and used for uptake studies and western blotting.

Timm-stain

Cryosections (25 µm) were cut in a coronal plane and mounted on slides (SuperFrost plus, Menzel-gläser, Braunschweig, Germany). Sections were dried and dampened for 6 hrs with H_2S gas (0,5% solution of Na_2S in MQ, adjusted to pH 7.3 using HCl) in a 10 liter desiccator jar.[260] Slides were stored overnight at -80˚C. Next day slides were subjected to Timm-stain according to Danscher.[261] In short, slides were immersed in a 0.5% v/v gelatin solution and dried. Subsequently sections were developed in a 6:2:1 mixture containing arabic gum, hydroquinone and citric acid-sodiumcitrate buffer, with 20 ml of 64.8 mM silver nitrate solution. Sections were counterstained with cresylviolet, dehydrated and coverslipped with malinol.

Immunohistochemistry

Immunostaining was performed at room temperature on de-waxed, 7 µm thick adjacent sections with commercially available affinity purified antibodies: guinea pig-anti-GLAST, guinea pig-anti-GLT1 (both 1:1000 and purchased from Chemicon Int. Inc. Temecula, CA, USA), rabbit anti EAAC1 (1:600, Biotrend Chemicals, Cologne, Germany), mouse-anti-vimentin (1:400, DakoCytomation, Glostrup, Denmark) and mouse-anti-GS (1:3200, BD Biosciences, Erenbodegem, Belgium). For each time point all sections were stained simultaneously. After rehydration, sections were placed in 0.01 M Sodium Citrate buffer (pH 6.0) and subjected to microwave treatment (7 min 650W, 5 min 350W) for antigen retrieval. Next sections were immersed in 0.3% H_2O_2 in Tris buffered saline (0.01M Tris, 0.9% NaCl, 0.2% TritonX100 (TBS_{TX})) for quenching of endogenous peroxidase activity. Sections were pretreated with 5% low fat dry milk (for GLAST and GLT1), 3% normal goat serum (for EAAC1), 3% normal horse serum (for Vimentin) or 3% fetal calf serum (for GS) for 1 hr at 37˚C. Primary antibodies were applied overnight at 4˚C, followed by incubation with biotinylated secondary antibodies (goat-anti-guinea pig, 1:500, Jackson Laboratories Inc, Cambridgeshire, UK; goat-anti-rabbit, 1:500, horse-anti-mouse 1:200, Vector Laboratories, Burlingame, CA, USA), for 1 hr. Immunoreactive staining was visualized using the avidin-biotin peroxidase method (Vectastain Elite, Vector, Brulingame, CA, USA) and 3,3'-diaminobenzidine (DAB) as chromogen.
For analysis of hippocampal morphology, representative brain sections (interval of approximately 100 µm) were stained with cresylviolet.
Immunohistochemistry was analyzed by visual inspection by two independent blinded observers. For each hippocampal subregion, sections were ranked according to their staining intensity. Nissl staining was also analyzed by visual inspection.

In situ hybridization

Partial cDNA was expressed in a pBluescript KS(+) vector and verified by sequence analysis. From human EAAT1, nucleotides 542-1242 (gi:472828, 88% identity with rat), from human EAAT2 nucleotides 1263-1875 (gi:498250; 88% identity with rat) and from mouse EAAC1 nucleotides 1354-1763 (gi:66789000; 90% identity with rat). After linearization and purification on agarose gel, riboprobes were prepared using T7 and T3 RNA polymerase by *in vitro* transcription and labeled using [^{33}P]-UTP (ICN Biomedicals, Irvine, CA, USA).

In situ hybridization was carried out on 7 µm paraffin sections. Sections were de-waxed, and deproteinized (30 min 10 µg/ml proteinase K treatment at 37˚C). Then sections were fixed in 2% PFA (4 min room temperature (RT)). Positive charge on the slides was blocked by acetylation for 10 min (0.25% acetic anhydride in 0.1 M triethanolamine, pH8.0). Just before hybridization sections were permeabilized with 0.1% TritonX100. Sections were then hybridized overnight at 55˚C with the [^{33}P]-labeled antisense probes (3*10^{6} cpm/slide) in 100 µl hybridization mix (containing 2x standard saline citrate (SSC), 50% deionized formamide, 10% dextran sulphate, 1x Denhardt's solution, 5 mM EDTA and 10 mM phosphate buffer, pH8.0). After hybridization, slides were placed in 5x SSC for 30 min at 65˚C. Two high stringency washes in 50% formamide in 2x SSC for 30 min at 65˚C were intermitted with a RNAseA treatment for 15 min at 37˚C (20 µg/ml in 0.5 M NaCl, 10 mM Tris and 5 mM EDTA, pH 8.0). Subsequently sections were washed in 2x SSC and 0.1x SSC for 15 min at RT, dehydrated in graded ethanol with 0.3 M ammoniumacetate and air dried. Slides were exposed to autoradiography films (Bio-Max MR, Kodak, Rochester, NY, USA) to visualize radioactivity.

For quantification purposes a graded [^{14}C] scale was exposed to every film. Exposure times were selected with grey values within the linear detection range. ImageJ (NIH) was used to quantify the mRNA expression. Films were calibrated using the [^{14}C]-scale. For GLAST and GLT1, mean gray values of different regions of interest were measured (CA1, CA3, infra- and suprapyramidal blade of dentate gyrus (DG) and the hilus of the DG) and corrected for background (four measurements in the corpus callosum). The EAAC1 images were thresholded to select stained cell bodies and the mean values above threshold were measured from pyramidal cells of the CA1, CA3 and granule cells from the infra- and suprapyramidal blade of the DG.

Glutamate uptake in hippocampal prisms

Hippocampi were dissected from their hemisphere. On a tissue chopper (McIlwain, Gomshal, UK) 250 µm slices were made, first in one orientation and than at a 90˚ angle. After equilibration for 15 min in Na^+-HT buffer (133 mM NaCl, 5mM KCl, 1.2 mM $MgSO_4$, 1mM KH_2PO_4, 0.75 mM $CaCl_2$, 10 mM Hepes, 5 mM Tris, pH 7.4) chops were washed 3 times. At the last wash-step half of the chops were resuspended in choline-HT buffer (NaCl replaced by equimolar choline chloride). After the last wash, buffer was removed and a 1:1 v/v suspension was made of chops and the appropriate buffer. 10 µl aliquots of chops were transferred to flat-bottomed 2 ml eppendorf tubes containing either Na^+- or choline-HT buffer. The chops were allowed to settle for 10 min at 37˚C, then uptake was initiated by adding [^{3}H]-glutamate (final

concentration 1 μM, 0.024 μCi, ICN Biomedicals, USA). After 1 minute, the reaction was stopped by adding 1.5 ml ice-cold Na^+ of choline-HT buffer and tubes were placed on ice. Chops were harvested using a Brandel cell harvester M24R on Whatman glass fiber paper GF/B under vacuum. Filters were rinsed 5 times with 2 ml ice-cold Na^+-HT buffer. Radioactivity on the filters was quantified by liquid scintillation counting. All determinations were performed in triplicate. Net high affinity, Na^+-dependent uptake was calculated by subtracting respective Na^+-free values and was expressed as pmol/10μl chops/min. Na^+-dependent uptake was linear for at least 5 min.
Part of the chopsuspension was homogenized and protein content was determined by the BCA Protein Assay kit (Pierce, Rockford, USA) using bovine serum albumin as a standard.

Western blot analysis

Homogenized hippocampal protein (from chop-assay) was solubilized in sample buffer (final concentration 0.001% w/v bromophenolblue, 5% v/v β-mercaptoethanol, 10% v/v glycerol, 2% w/v SDS, 62.5 mM Tris-HCl, pH 6.8), was separated on 10% SDS-PAGE gels and was transferred to polyvinylidene difluoride membranes (PVDF, Amersham, Buckinghamshire, UK) by semi-dry blotting for 1.1 hr at 400 mA. Membranes were blocked for 1 hr at RT with 5% v/v normal goat serum (NGS) and 5% w/v low fat dry milk in 50 mM Tris-buffered saline containing 0.1% v/v Tween-20 (TBSt). Primary antibodies (see section immunohistochemistry) were applied overnight at 4˚C at the following dilutions: GLAST 1:5000, GLT1 1:20000, EAAC1 1:500 in TBSt with 10% v/v NGS. Membranes were subsequently incubated with alkaline phosphatase-conjugated secondary antibodies (1:2000 in TBSt + 10% v/v NGS). Immunoreactivity was visualized using enhanced chemofluorescence substrate (ECF, Amersham, Buckinghamshire, UK) on a Fluor-S MultiImager (BioRad, Hercules, CA, USA). Fluorescence was quantified with a Quantity One software package (BioRad, Hercules, CA, USA). Control experiments without the primary antibody did not show any staining (not shown). Linearity was tested beforehand using serial dilutions of a rat hippocampus homogenate.

Statistics

Data are expressed as mean ± SEM. One-way ANOVA (analysis of variance) combined with a *posthoc* Bonferroni test for multiple comparison (SPSS software package), was used to test significant differences between groups. P-values < 0.05 were considered significant.

Results

Seizures

After pilocarpine injections, rats immediately showed wet-dog shakes, followed by mouth and facial movements within minutes and this progressed within 15 minutes to

head nodding and forelimb clonus. These partial seizures became more general after 30-45 min, when rearing and falling and eventually after 1.5 hrs loss of postural control were observed in the animals. Animals had intermitted periods of generalized seizures for 1 hr before being sedated with diazepam.
From the 68 animals receiving the full treatment, 48 animals reached stage 6 seizures[259] (71%, +SE group), whereas 20 animals did not (29%, –SE group). From the animals treated with pilocarpine, 13 animals died during or within 3 days (12 +SE, 1 –SE). Spontaneous recurrent seizures (SRS) were not seen before 15 weeks after SE induction. From the +SE animals in the 19-weeks group, 8/18 animals developed SRS and 1/10 animals in the –SE group. Observation was done daily for about 1 hr during the light phase and during handling.

Hippocampal morphology

Cresylviolet (Nissl) staining was used to identify gross morphological alterations. No change in cell number, especially in the hippocampus, was observed by visual examination at the four different time-points after induction of SE compared to the control groups (fig. 1). In the 19-weeks group we examined mossy fiber sprouting using Timm-stain. As Timm-stain cannot be performed on PFA fixed and paraffin embedded tissue, a different group of animals was used and sacrificed at 19 weeks after pilocarpine induced SE. Five out of eight animals from this +SE group had developed SRS. The pattern of mossy fibers of the eight +SE animals was identical to that of –PC animals, thus mossy fiber sprouting was not observed (fig. 2).

Expression of GLAST

GLAST immunoreactive (IR) staining of sections of –PC animals from the 2 week group resulted in a patchy staining of astrocytes in the neuropil throughout the hippocampus. The stratum oriens of CA1-3 and the outer molecular layer of the dentate gyrus were more intensely stained than the stratum radiatum and the hilus of the DG. The stratum lacunosum-moleculare was less stained and was clearly visible as a lighter band (fig. 1). The pyramidal cell layer and the granule cell layer were almost devoid of staining. Directly beneath the granule cell layer a small but very dark IR band was observed. The staining pattern of SHAM animals was not different from –PC animals in any aspect, and were therefore considered as one control group. Also no difference was seen between animals of different ages tested in this study, except that the stratum lacunosum-moleculare was less visible as a lighter band in the older age groups. The staining pattern for GLAST in other parts of the brain was as described previously.[179,251]
In SE animals 2 weeks after the inductions of SE, IR was more intense than in sections from controls. This was seen in the hippocampus as well as in general across the whole brain. In the hippocampus, this increase was most pronounced in the stratum oriens of the CA1 field, the molecular layer of the infrapyramidal blade of the dentate gyrus (DG) and in the stratum lacunosum-moleculare (SLM). In the latter region, control animals showed a light band just above the hippocampal fissure, whereas in +SE animals this band had the same intensity as the

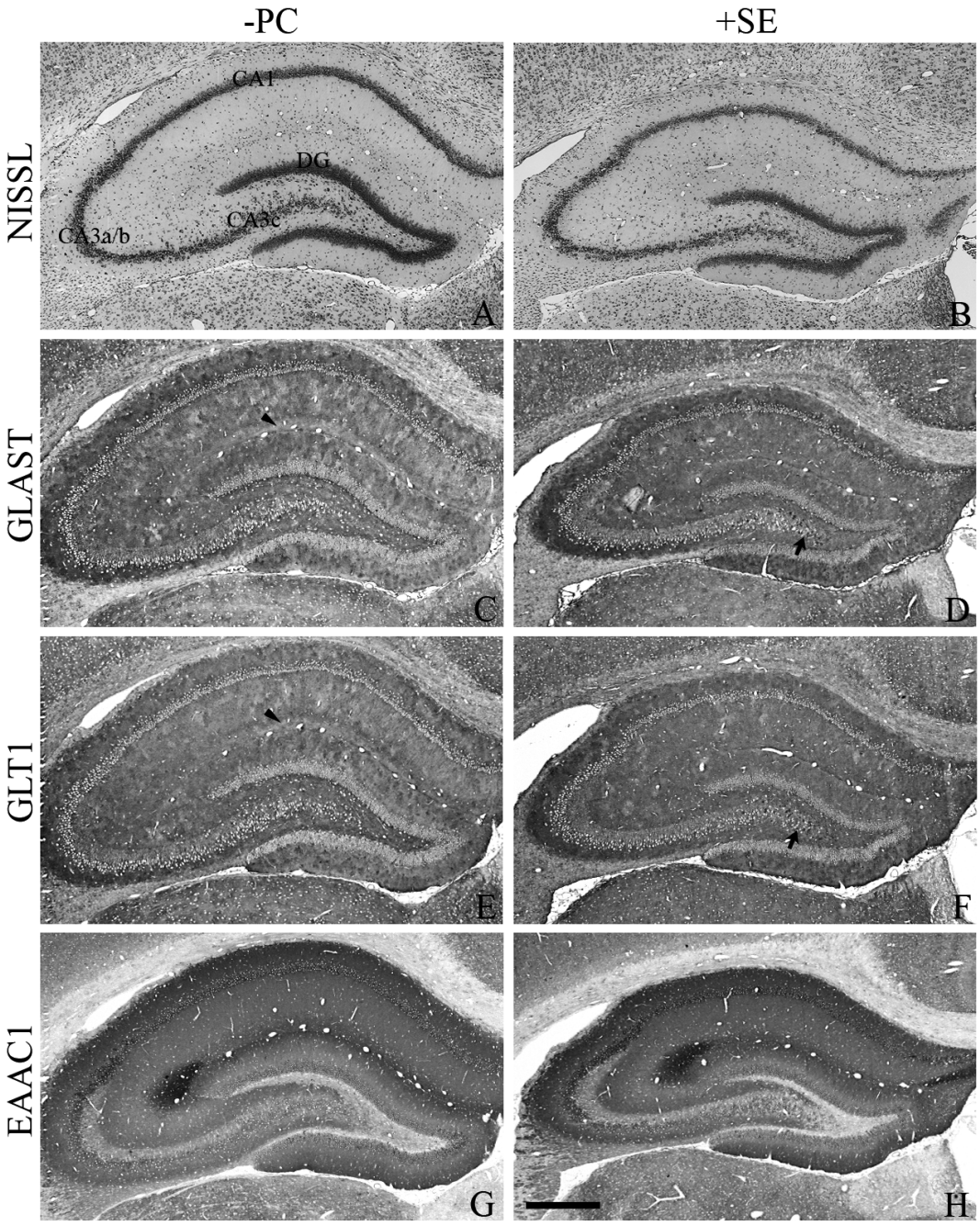

Figure 1. *Nissl-stain (A,B) and immunostaining for GLAST (C,D), GLT1 (E,F) and EAAC1 (G,H) in a control hippocampus (–PC: A,C,E,G) and 2 weeks after pilocarpine induced SE (+SE: B,D,F,H). Arrowheads indicate the stratum lacunosum-moleculare which is only lightly stained for GLAST and GLT1 IR in –PC animals. Arrows indicate the scattered darkly stained GLAST and GLT1 immunoreactive cells in the hilus in +SE animals. CA=cornu Ammonis, DG = dentate gyrus. Scale bar = 500 μm.*

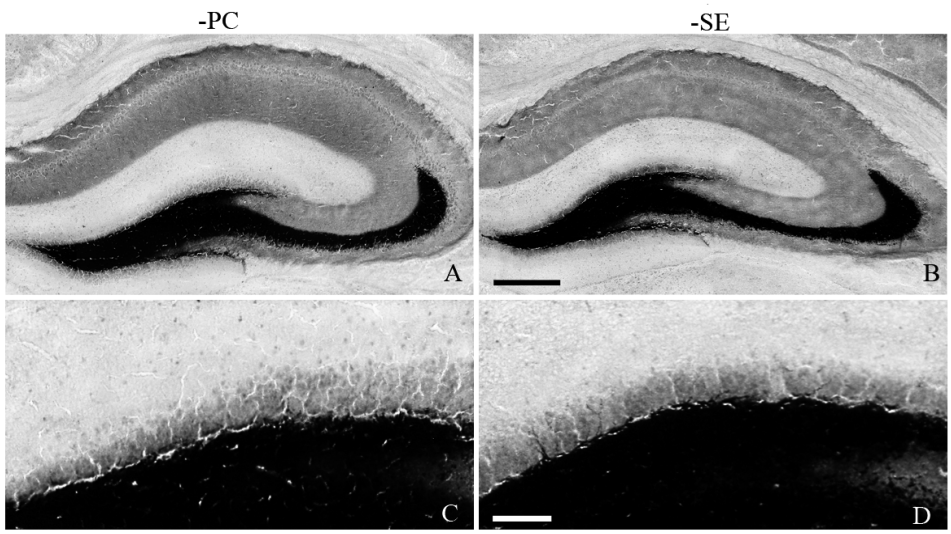

Figure 2. *Timm staining showing mossy fibers in the dentate gyrus. The pattern of mossy fiber staining is identical in –PC and +SE rats at 19 weeks after pilocarpine induced SE. Scale bar A,B = 500 μm, C,D = 100 μm .*

stratum radiatum (fig. 2). These changes were also seen in some animals 4 weeks after SE induction, but they were transient as they were absent in animals 8 and 19 weeks after SE induction.

Interestingly, animals 2 weeks after SE induction showed scattered, very darkly stained cells in the tip of the CA3c region, thus inside the hilus of the DG. These cells were not seen in animals sacrificed at any other time-point or in controls (fig. 3).

Total GLAST protein content, measured by semi-quantitative Western blot analysis in whole hippocampal homogenates from animals 19 weeks after induction of SE, showed no difference between the four experimental groups (fig. 5).

In situ hybridization for GLAST mRNA displayed a pattern similar to that described previously[252,262](fig. 6). A strong signal was detected in the cerebellum. Less, but still pronounced staining was present in the cortex and hippocampus. GLAST mRNA was scarcely present in the thalamus and some hypothalamic regions. Throughout the hippocampus a diffuse labeling was visible. The region in close proximity of pyramidal cell bodies was more intensely labeled as was the dentate gyrus. This pattern was recognizable in all experimental groups and time-points tested. Thus no changes were observed in GLAST mRNA expression in any of the animal groups tested.

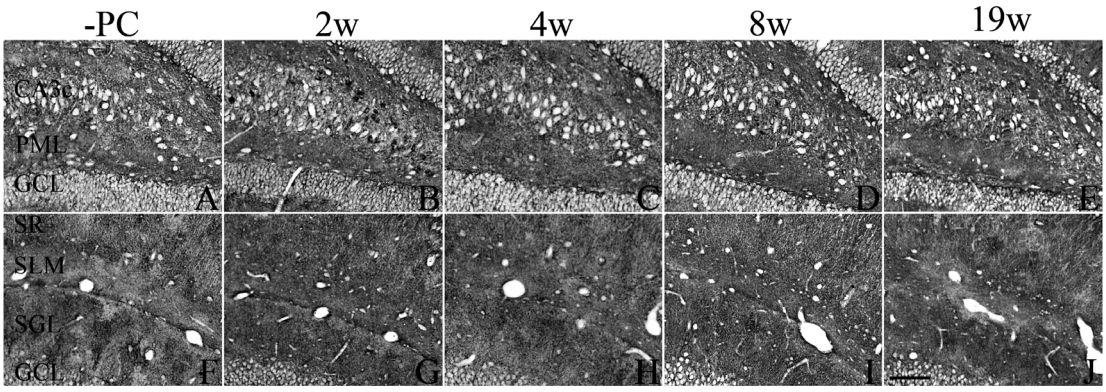

Figure 3. *GLAST immunostaining in the hilus (A-E) and CA1 area (F–J) of a control animal (–PC) and 2, 4, 8 and 19 weeks after induction of SE (+SE). CA3c = cornu Ammonis subfield 3c, PML = polymorphic layer, GCL = granule cell layer, SR = stratum radiatum; SLM = stratum lacunosum-moleculare; SGL = supragranular layer. Scale bar = 100 μm.*

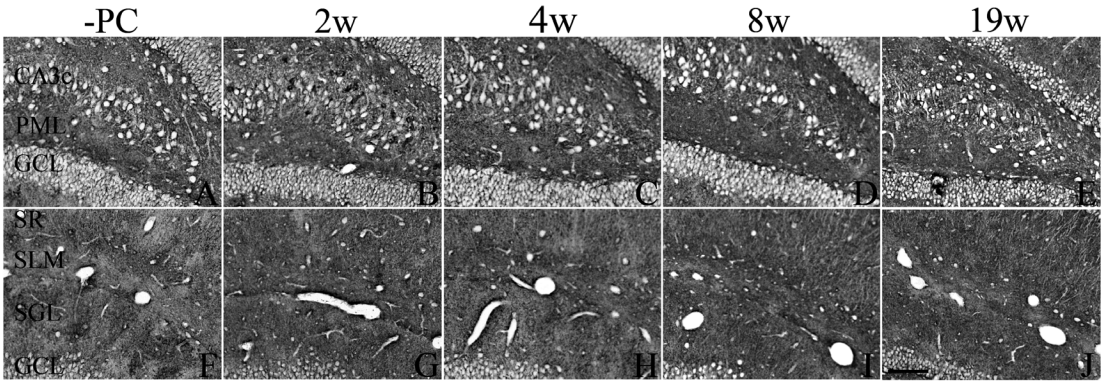

Figure 4. *GLT1 immunostaining in the hilus (A-E) and CA1 area (F-J) of a control animal (–PC) and 2, 4, 8 and 19 weeks after induction of SE (+SE). CA3c = cornu Ammonis subfield 3c, PML = polymorphic layer, GCL = granule cell layer, SR = stratum radiatum; SLM = stratum lacunosum-moleculare; SGL = supragranular layer. Scale bar = 100 μm.*

Expression of GLT1

Immunostaining of GLT1, showed intense staining in the neuropil of the hippocampus with a mostly patchy appearance, as described previously.[179,263] The stratum oriens and the dentate gyrus (hilus, polymorphic layer, stratum granulosum and stratum moleculare) were more intensely stained. The stratum lucidum was visible as a lighter band between stratum pyramidale and the stratum radiatum in the CA3 field. This staining pattern was identical in control rats (SHAM and –PC) at all four time-points. Overall staining was the same in controls compared to +SE animals (fig. 1). Some of the effects seen in GLAST staining were also seen in GLT1 immunostaining. Two weeks after induction of SE, the IR in the SLM was weaker in control animals than in +SE animals, where this layer was as intense as the stratum radiatum (fig. 4). As for GLAST, this was also a transient effect and was seen only in some of the animals 4 weeks after induction of SE, but not in animals 8 and 19 weeks after induction of SE.

As in GLAST staining, a few intensely stained cells were seen in the tip of CA3c in the hilus of the DG in sections of 2 weeks +SE animals (fig. 4).
Total hippocampal GLT1 protein was measured by semi-quantitative Western blot (fig. 5) and was not changed in 19 week +SE animals compared to controls.
The expression patterns of GLT1 mRNA was consistent with previous reports.[252,262] The riboprobes used to detect GLT1 mRNA intensely labeled cortical regions, including the hippocampus (fig. 6). Major fiber tracks (corpus callosum, optic tract, the internal capsule) were not labeled. Besides a diffuse overall labeling in the hippocampus, GLT1 mRNA was pronounced in a distinct band, in and around the principal cell layers and darkest in the CA3 field. Visual inspection as well as quantification of the 2 week time-point of the *in situ* hybridization revealed no differences in expression of GLT1 mRNA between the groups.

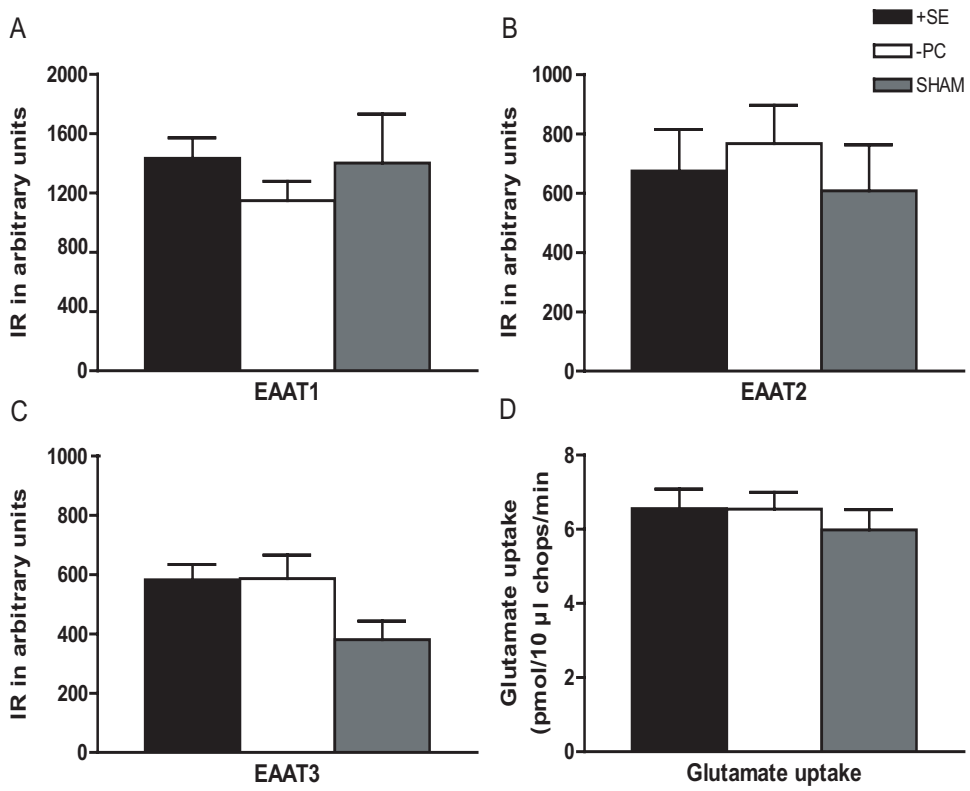

Figure 5. *Western blot analysis of GLAST (A), GLT1 (B) and EAAC1 (C) protein in hippocampal homogenates from animals 19 weeks after pilocarpine induced SE. GLAST, GLT1 and EAAC1 IR was not different between +SE animals and controls. (D) Glutamate uptake in hippocampal prisms. Uptake was not different between the 3 different animal groups. +SE = animals with SE after pilocarpine injection, –PC = control animals that received full treatment except pilocarpine. SHAM= control animals that received all injections with saline.*

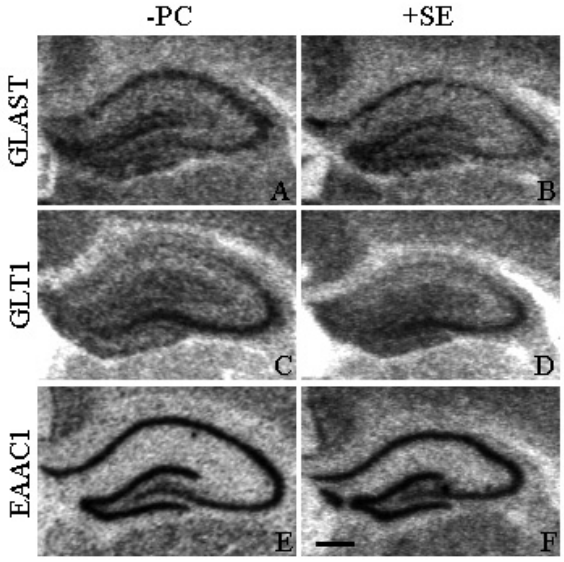

Figure 6. *Expression of GLAST (A,B), GLT1 (C,D) and EAAC1 (E,F) mRNA in control hippocampus (-PC) and 2 weeks after the induction of SE (+SE). Scale bar = 500 μm.*

Expression of EAAC1

Intense immunostaining was present in all neurons in the hippocampus. EAAC1-IR was prominently found in the stratum pyramidale of CA1-3 and less intense in the cell bodies of the granule cells of the DG and in cells in the hilar region.[179,253] This pattern was identical in SHAM and –PC animals at all time-points. No detectable changes were found in the pattern of EAAC1 expression or in the intensity of staining in the different subfields of the hippocampus of animals at the different time-points after pilocarpine induced SE (fig. 1).

Total EAAC1 protein of hippocampal homogenates was measured by semi-quantitative Western blot and was identical in the four different experimental groups at 19 weeks after the induction of SE (fig. 5).

EAAC1 mRNA was prominently present in the hippocampal area. In control animals, a high signal was present in the stratum pyramidale of CA1-3 and in the stratum granulosum of the DG (fig. 6). This was identical to previously reported expression patterns.[252,264] Visual inspection revealed no difference between controls and pilocarpine treated animals at the four different time-points measured.

Glutamate uptake

At 19 weeks after SE, hippocampal prisms of +SE rats (n=8) had an uptake of 6.55 ± 0.53 pmol/10µl/min ± SEM. –PC rats (n=8) had an uptake of 6.55 ± 0.45 pmol/10µl/min and SHAM rats (n=4) had an uptake of 5.98 ± 0.55 pmol glutamate/10µl prisms/min (fig. 5). Glutamate uptake of –SE rats was not measured. Thus, glutamate uptake 19 weeks after SE did not differ significantly in the three experimental groups.

Other glial parameters

To investigate whether the changes in GLT1 and GLAST staining in the animals 2 weeks after pilocarpine induced SE are associated with reactive gliosis, we analyzed vimentin staining in adjacent sections. Vimentin IR was detected in the tip/crest of the polymorphic layer of the DG and in the SLM of the CA1 and in animals 2 weeks after induction of SE, but not in control animals (SHAM, –PC; fig. 7). Subsequently, we analyzed GS, another glial marker, which is associated with glutamate uptake. GS IR showed no difference between control rats and +SE rats 2 weeks after induction of SE (fig. 7).

Glutamate transporters in –SE rats

Interestingly, –SE rats, that did not reach stage 6 seizures during the treatment with pilocarpine, also showed the increased expression of GLAST and GLT1 in the stratum lacunosum-moleculare and the few heavy stained GLAST or GLT1 stained cells in the hilus. However, these changes were not visible after 2 weeks, as in the +SE animals, but after 4 weeks (fig. 8). These changes were also transient, as they were not present anymore in animals of 8 and 19 weeks after pilocarpine induced SE. The delayed pattern of changes was also found in vimentin IR. Vimentin IR was faintly present in the polymorphic layer of the DG in 2 week animals, but reactive glia cells were clearly present in animals sacrificed 4 weeks after SE induction, in the stratum lacunosum-moleculare and in the hilus of the DG. GS immunostaining was not different from control animals in –SE animals 2 and 4 weeks after pilocarpine induced SE (fig. 8).

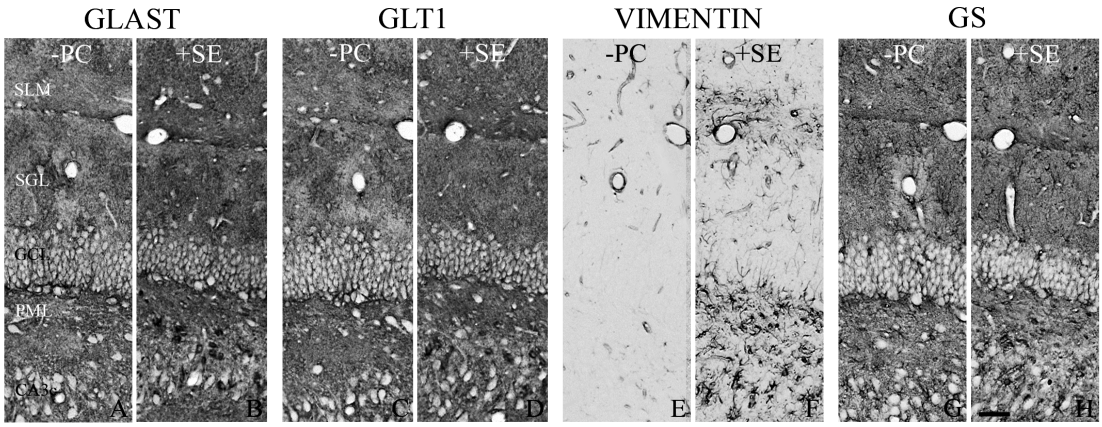

Figure 7. *Comparison of GLAST, GLT1, GS and Vimentin immunostaining in adjacent sections of the hilus of a control animal (–PC) and a SE animal (+SE) 2 weeks after pilocarpine injection. SLM = stratum lacunosum-moleculare, SGL = supragranular layer; GCL = granule cell layer; PML = polymorphic layer; CA3c = cornu Ammonis subfield 3c. Scale bar = 50 μm.*

Discussion

The major findings of this paper are that in a mild juvenile pilocarpine model, which shows no neuronal cell death and mossy fiber sprouting, transient changes can be observed in the expression of two glial glutamate transporters GLAST and GLT1. An increase in GLAST and GLT1 IR was found in +SE rat 2 weeks, but not 4, 8 and 19 weeks after SE induction. This increase was found in the stratum lacunosum-moleculare and in particular cells in the hilus of the dentate gyrus for both GLAST and GLT1, and GLAST expression was slightly increased in the whole hippocampus.
The increase in GLAST and GLT1 staining in the stratum lacunosum-moleculare is most likely due to an increased expression in glial cells. Two weeks after SE we also found vimentin staining is this layer, suggesting reactive gliosis. It is possible that all these changes reflect a transient increase in glutamatergic transmission in this layer,

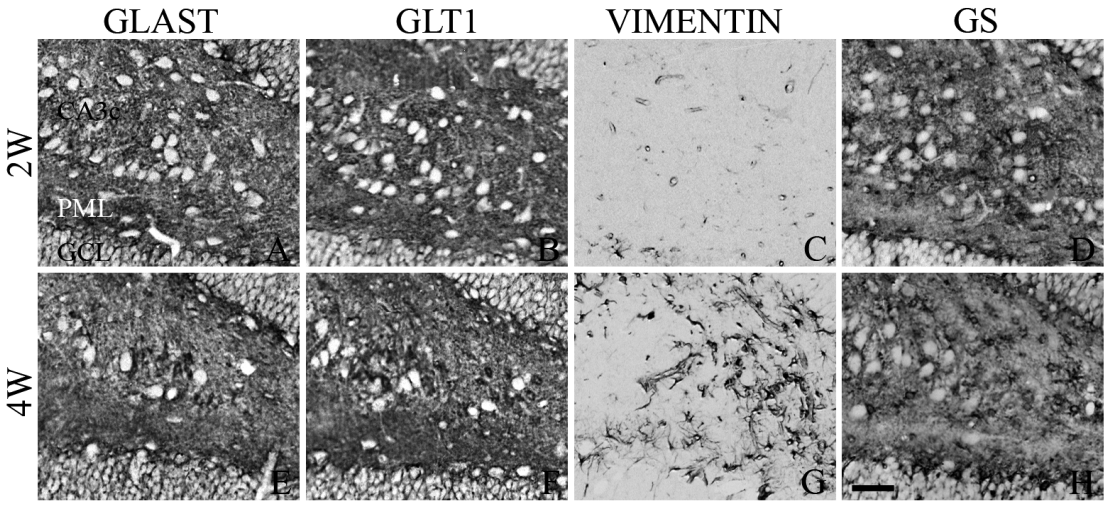

Figure 8. *GLAST, GLT1, GS and Vimentin immunostaining in the hilus of pilocarpine treated animals without a full SE (–SE group) 2 and 4 weeks after treatment. CA3c = cornu Ammonis subfield 3c, PML= polymorphic layer, GCL = granule cell layer. Scale bar = 50 μm.*

but they might also be a reaction to the intense firing of neurons during the pilocarpine induced SE. The latter possibility predicts that the effects would be more pronounced at shorter time-points after the induction of SE.

The identity of the cells in the hilus which are intensely stained for GLAST and GLT1 is unclear. The fact that in the hilus similar changes in staining patterns were observed for the glial transporters GLAST and GLT1 indicates that these intensely stained cells are a specific subset of glia cells. Possibly these cells are reactive glia, because in +SE animals in the same hilar region we found scattered vimentin immunoreactive glia. However, we cannot rule out that GLAST and GLT1 are expressed in distinct glial populations or even in some neurons, a phenomenon which has been shown under pathological conditions.[124,265] Presently we are performing double label immunofluorescence experiments to resolve this issue.

We found no change in GS IR in the hippocampus of animals 2 weeks after pilocarpine induced SE. In human TLE with hippocampal sclerosis (HS), a decreased hippocampal GS expression was only present in areas with neuron loss and was not found in TLE hippocampi without HS.[126] The lack of changes in hippocampal GS expression in the pilocarpine model appear to be consistent with the fact that we did not find evidence for hippocampal neuron loss.

Rats that do not respond to lithium-pilocarpine with a full SE (-SE) are sometimes proposed as suitable treatment controls. However, in the present study we show that in –SE animals the same transient changes in GLAST, GLT1 and vimentin staining occur as in +SE animals, only somewhat later at 4 weeks after pilocarpine treatment. Moreover, 19 weeks after pilocarpine treatment one of the –SE animals showed SRS. Thus, it appears that in –SE animals a pathologic process similar to that in +SE animals takes place, but at a somewhat slower pace.

Comparison with other epilepsy models

Animals in our study did not show obvious neuron death or mossy fiber sprouting, but a large percentage did develop SRS. We choose to use a mild juvenile pilocarpine model, induced by a moderate dose of pilocarpine (40 mg/kg). This dose was used to avoid massive cell death which has been reported in several other studies. Our data show that the neuron death caused by the prolonged pilocarpine-induced SE is not a prerequisite for the induction of epileptogenesis. This observation is consistent with a number of studies reporting no cell loss after pilocarpine induced SE (+ or – lithium) around P21.[266,267] However, other studies on the pilocarpine model report cell loss and other features of hippocampal sclerosis including mossy fiber sprouting (e.g.[98,268-271]).
It is difficult to compare our data with other animal studies. In the adult and juvenile pilocarpine model only EAAC1 expression has been studied. EAAC1 mRNA was found to be increased in individual granule cells during the latent phase as well as the chronic phase.[257,258] GLAST and GLT1 expression have not been studied at all in the pilocarpine model. The expression of the 3 major glutamate transporters has been studied in a variety of other epilepsy models, but the results are not very consistent. For instance in the rat kainate (KA) model, GLAST mRNA in the hippocampus was found to be increased after 6 days,[272] but decreased after 60 days.[273] After the induction of SE by electric stimulation, GLAST expression was also found to be decreased in the hippocampus.[274] Changes in GLT1 expression in the hippocampus range from unchanged in the KA model 2 days after SE[275], via decreased after 60 days,[273] to increased in the dentate gyrus after electrically induced SE.[274] A similar variability is fouond in hippocampal EAAC1 expression in the various models [274-276]
Our data in the juvenile pilocarpine model clearly show the importance of longitudinal expression studies. To elucidate the role of glutamate transporters in epileptogenesis it will be important to perform similar studies in different epilepsy models.

Comparison with human epileptic tissue

The rat model used in this study might be best compared with human TLE patients without hippocampal sclerosis (non-HS), as we found neither obvious neuron loss nor mossy fiber sprouting. In these patients EAAT2/GLT1 expression has been reported to be increased compared to autopsy controls.[124] Moreover, these patients have unaltered GS expression in the hippocampus.[126] However, it should be emphasized that TLE patients have suffered from TLE for many years, whereas in our rat model the animals have just developed spontaneous recurrent seizures.
In TLE patient with hippocampal sclerosis (HS), a moderate general decrease in EAAT1/GLAST expression compared to non-HS- tissue or to autopsy controls was reported.[124] Both mRNA and protein were decreased, which was most pronounced in the CA4, the PML and the SGL,[124] whereas another study found no changes in EAAT1.[125] More dramatic changes were found in hippocampal EAAT2 expression in HS patients. Both studies reported a large decrease in EAAT2 in areas of neuron loss in HS patients, compared to the non-HS patients,[124,125] and report also the presence of EAAT2 mRNA in neurons.[124] In HS patient an increase in EAAT3 expression was found in the surviving neurons.[124,125] TLE patients with HS show a pronounced

reduction in glutamine synthetase in areas of severe neuronal cell loss, compared to non-HS or autopsy controls.[126,127] Consistent with the lack of neuron loss, we did not find changes in GS expression 2 weeks after pilocarpine induced SE.

Concluding remarks

In this mild model of TLE, without neuronal cell loss or mossy fiber sprouting, transient upregulation of GLAST and GLT1 was observed in particular areas of the hippocampus. These were seen in +SE animals at 2 weeks after induction of SE and at 4 weeks after SE in –SE animals. Possibly, these changes reflect temporary changes in glutamatergic transmission and may be part of the complex mechanism leading to epileptogenesis. The functional implications of the transient increase in glial glutamate transporters are unknown and need to be further investigated. Obviously, these changes are only one factor in a complicated mechanism of epileptogenesis. To learn more, expression changes of e.g. proteins active in neurotransmission (glutamatergic, GABAergic) or ion channels responsible for membrane potential need to be investigated as well.

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6

GS expression in leukocytes of pilocarpine treated rats

Ineke W.M Bos, Elly J.F. Vereyken, Marina de Wit,
Marije G. Rensen-de Leeuw, Onno van Nieuwenhuizen,
Pierre N.E. de Graan

Abstract

Temporal lobe epilepsy (TLE) is a common epileptic disorder, in which excitotoxicity due to impairments in the glutamate-glutamine cycle has been implicated in the pathogenesis. The glia specific enzyme glutamine synthetase (GS) plays a key role in the glutamate-glutamine cycle by converting glutamate into glutamine. In the hippocampus of TLE patients with hippocampal sclerosis GS expression is decreased in areas with severe neuron loss. Moreover, we found decreased leukocyte GS expression in epileptic children, directly after the initial diagnosis and 1.5 years later, regardless of treatment with antiepileptic drugs. In this study we investigate longitudinal changes in GS expression in the hippocampus and blood leukocytes of P21 rats after the induction of SE with lithium-pilocarpine. In this mild juvenile pilocarpine model, leukocyte GS expression was 2, 4, 8 and 19 weeks after pilocarpine induced SE compared to controls. Two weeks after SE, a time-point at which glutamate transporter and vimentin expression are altered, hippocampal GS immunoreactivity was not different from controls. Both control animals and pilocarpine treated rats showed increased levels of leukocyte GS expression at 2 weeks after treatment, probably induced by lithium.

As we found no changes in hippocampal GS expression, this mild juvenile pilocarpine model appears not to be suitable to study a correlation between brain and blood GS expression. Other animal models for TLE, particularly those with severe neuron loss, need to be examined.

Introduction

Temporal lobe epilepsy (TLE) has been associated with excitotoxicity due to increased levels of glutamate.[116] Microdialysis revealed that TLE patients have a sustained increase in hippocampal extracellular glutamate during and after a seizure.[118] Extracellular glutamate is removed by excitatory amino acid transporters (EAATs),[11] predominantly by EAAT2 expressed in astrocytes.[3] Glutamate taken up by astrocytes is converted into glutamine by the glia specific enzyme glutamine synthetase (GS; EC 6.3.1.2).[14] Glutamine, which is non-toxic, is transported back to neurons and can be re-used as neurotransmitter after enzymatic conversion to glutamate. This process is known as the glutamate-glutamine cycle.[2] A number of studies indicate a role of GS in seizures and epilepsy. Animals studies show a reduced brain GS expression,[277-279] induction of seizures by inhibiting GS activity[246] and a reduction of brain GS activity after antiepileptic drug treatment.[280] Moreover, the human epileptic hippocampus, resected to remove the seizure focus in TLE patients, is shown to have a large decrease in GS protein and activity.[126,127]

Recently we have shown a reduction in blood leukocyte GS expression in young epileptic children (Bos *et al.*, chapter 4), indicating that GS expression is a potential marker for epilepsy. A first step to test the validity of GS as marker for epilepsy is a longitudinal correlative study on blood and brain GS expression in an animal model for TLE. We used the juvenile pilocarpine model, in which lithium/pilocarpine treatment of 21-day-old rat pups induces a status epilepticus (SE), which after a latent phase, develops into a chronic phase with spontaneous recurrent seizures.[98] In a previous study we showed that 45% of the animals developed spontaneous recurrent seizures (SRS) at 19 weeks after pilocarpine induced SE (Bos *et al*., chapter 5). Two weeks after induction of SE these animals show a transient increase in glial glutamate transporter proteins GLAST and GLT1 in particular areas of the hippocampus. At this time-point the same areas also show increased expression of the reactive glia marker vimentin (Bos *et al.*, chapter 5).

The aim of this study is to analyze GS expression in leukocytes after induction of a status epilepticus (SE) in young rats by lithium/pilocarpine injection (Bos *et al*., chapter 5). At various time-points after the pilocarpine-induction of status epilepticus, during the latent and the chronic phase, leukocytes were isolated and GS levels in these cells were determined using quantitative PCR.

Materials and Methods

Animals

Male Wistar rats (Charles River Laboratories, Schulzfeld, Germany) arrived at postnatal day 8 (P8) in litters of 10 pups with a foster mother. Rats were weaned and housed individually at P21. Animals were housed in a temperature and humidity controlled room with tap water and standard rat chow freely available and lights on between 7AM and 7PM. The experimental procedures were approved by the Ethical

Committee for Animal Experiments of Utrecht University.
At P20, rats were injected with lithium-chloride (Merck, Darmstadt, Germany) 3 mmol/kg intraperitoneally (i.p.). At P21, 18-20 hrs after lithium treatment, animals were injected with methyl-scopolamine i.p. (1 mg/kg, Sigma, St. Louis, MO, USA). Rats were placed in individual plastic cages for behavioral observation. Thirty minutes later, pilocarpine (40 mg/kg, Sigma, St. Louis, MO, USA) was administered subcutaneously. The behavior of the animals was observed and classified in six stages according to Racine (1972[259]). After one hour of SE rats were injected with diazepam (4 mg/kg i.p., Centrafarm Services BV, Etten-Leur, the Netherlands) to reduce mortality. Control animals were handled and housed in the same manner. The control group (–PC), received full treatment except that pilocarpine injections were replaced by an equal volume of saline.

Immunohistochemistry

Immunostaining was performed as described earlier (Bos *et al*. chapter 5) on 7 µm paraffin sections using mouse-anti-GS (1:3200, BD Biosciences, Erenbodegem, Belgium) as primary antibody, followed by incubation with a biotinylated secondary antibody; horse-anti-mouse (1:200, Vector, Burlingame, CA, USA). Immunoreactive staining was visualized using the avidin-biotin peroxidase method (Vectastain Elite, Vector, Brulingame, CA, USA) using 3,3'-diaminobenzidine (DAB) as chromogen.
Adjacent brain sections were stained with cresylviolet (Nissl-stain) to show hippocampal morphology and to examine neuron loss.

Isolation of leukocytes mRNA

At 2, 4, 8 or 19 weeks after pilocarpine SE rats were sacrificed by i.p. injections of 300 mg/kg pentobarbital and blood was collected by cardiac-puncture using a syringe filled with heparin. A separate group of rats was sacrificed 19 weeks after pilocarpine induced SE by unanaesthetized decapitation and trunk blood was collected in heparin containers.
Blood was mixed 1:1 with 10 mM phosphate buffered saline, pH 7.4 (PBS), and centrifuged 2200 rpm, 20min, at 4˚C. Buffycoat was removed and mixed with NH_4Cl lysis-solution. After full lysis of erythrocytes, leukocytes were centrifuged (2200 rpm for 5 min at 4˚C), washed in PBS and resuspended in 50 µl PBS. Leukocytes were lysed in 1 ml TRIzol (Gibco Brl, Life Technologies, Gaithersburg, Maryland, USA) and stored at -80°C until RNA isolation according to manufacturer's protocol.

Quantitative PCR

cDNA synthesis was preceded by a DNase treatment (DNaseI, Invitrogen, Paisley, Scotland) according to the manufacturer's protocol. RT-PCR was carried out using Superscript II (Invitrogen) and oligo-dT primers according to the manufacturer's protocol, but without DTT and RNA-guard.
The qPCR reaction was performed as described earlier (Bos *et al*, chapter 4). In short, qPCR was carried out using the LightCycler (Roche, Basel, Switzerland) and the Fast Start SYBRgreen master mix (Roche) according to manufacturer's protocol. Primers

used for GS were FW-GGAGGAGAATGGTCTGAGGT, REV-AGCGGAAAAGTC GTTGATGT, for succinate dehydrogenase subunit A (SDHA) FW-TTACAAGGTG CGGATTGATG, REV-AGGAACGGATAGCAGGAGGT, for cyclophilin FW-ATG TGGTCTTTGGGAAGGTG, REV-GAAGGAATGGTTTGATGGGT (5'-3'; Isogen Biosciences BV, Maarssen, the Netherlands, HPLC purified). All appropriate controls were carried out to optimize the PCR reaction and specificity was verified by melting temperature determination at the end of each run. Cycles of threshold (Ct) for each sample were determined using the second derivative maximum (Light Cycler Software, version 3.5, Roche). Gene expression was calculated as normalized ratio (Rn), which is a measure for the amount of cDNA present in a sample relative to a calibrator sample measured in the same qPCR experiment and normalized to a reference gene. A pool of leukocyte cDNAs from different donors was used as calibrator. Succinate dehydrogenase subunit A (SDHA[244]), β-Actin and cyclophilin A[245] were used as reference genes. The expression of SDHA and cyclophilin was highly correlated ($r=0.77$, $p<0.001$).

All samples were analyzed in duplicate and reported as mean. Data are expressed as mean ± SEM. One-way ANOVA (analysis of variance) combined with a *posthoc* Bonferroni test for multiple comparison and Students t-test (GraphPad Prism), were used to test significant differences between groups. P-values <0.05 were considered significant.

Results

GS expression in leukocytes

In preliminary experiments we tested the expression of GS mRNA, protein and activity. RT-PCR analysis detected GS mRNA in rat leukocytes, and western blotting revealed GS immunoreactivity (data not shown). A GS enzyme activity assay[126] proved that GS was a functional enzyme in rat leukocytes (data not shown).

Leukocytes from control animals (–PC) 2 weeks after treatment (thus 5 weeks of age) had a significantly higher GS expression than control animals 4, 8 and 19 weeks after pilocarpine induced SE (fig. 1a, $p<0.001$). The normalized ratio (Rn) ± SEM of GS relative to SDHA expression was in the –PC group at 2 weeks after pilocarpine induced SE 6,44 ± 1.35 (n=3); at 4 weeks: 2.40 ± 0.54 (n=6); at 8 weeks: 1.44 ± 0.166 (n=4) and at 19 weeks: 1.585 ± 0.23 (n=15) (fig. 1a). Also +SE animals at 2 weeks after pilocarpine induced SE had a significantly higher Rn for GS (4.92 ± 0.91 n=3) than the +SE animals at later time-points (at 4 weeks: 2.82 ± 0.50, n=6; at 8weeks: 2.38 ± 0.75, n=5 and at 19 weeks: 2.08 ± 0.38, n=13; $p<0.001$).

However, GS expression of +SE animals was not different from control (–PC) animals at 19 weeks after pilocarpine induced SE (fig. 1b). The +SE group could be divided in animals suffering from SRS and animals that were not. The GS expression was not different between those two animal groups (+SE +SRS 2.05 ± 0.66, n=7; +SE-SRS, 2.10 ± 0.36, n= 6). –SE animals were only tested 8 weeks (n=5) and 19 weeks (n=9) after pilocarpine induced SE. Analysis of variance (ANOVA) showed no difference between the GS expression in leukocytes of the +SE group, the –SE group

and –PC group of the four different time-points tested. Similar results were obtained when normalizing GS expression data relative to cyclophilin instead of SDHA) (data not shown).

GS immunostaining

The Nissl stain showed no difference in cell number in the hippocampus between +SE and –PC animals (data not shown). Immunohistochemical staining of the glial enzyme GS in the hippocampus of –PC and +SE animals showed prominent immunoreactivity (IR) of the neuropil of the CA-fields and the dentate gyrus (fig. 2). No differences in expression pattern were seen between the experimental groups.

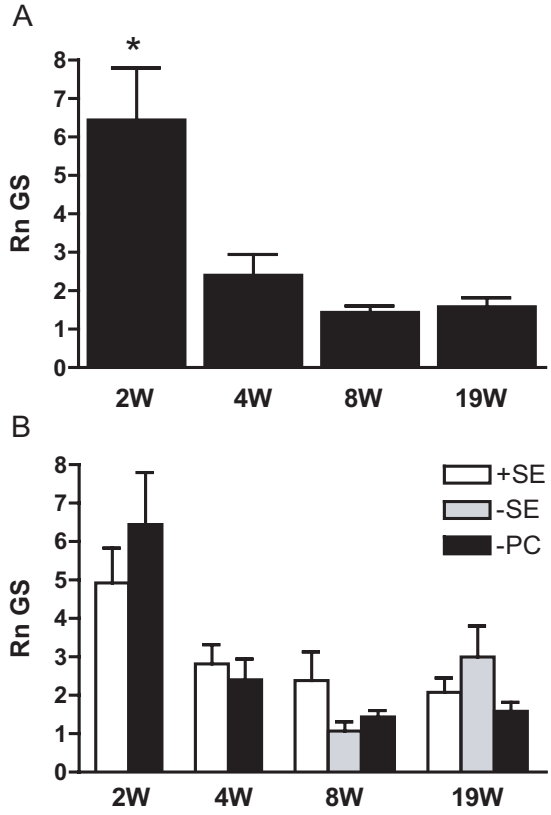

Figure 1.
A Normalized ratio (Rn) of GS expression relative to SDHA in leukocytes of –PC rats at four different time-points after the induction of SE. The 2 week group (control rats of 5 weeks old), showed a higher GS expression than rats of older ages .
** p<0.001*
B: Normalized ratio (Rn) of GS expression relative to SDHA in leukocytes from rats of the juvenile pilocarpine model. At all four time-points measured, +SE and –SE animals had a normal GS expression in their leukocytes. As in A, animals at 2 weeks after pilocarpine induced SE had a higher expression of leukocyte GS than at other time-points (p<0.001).
W = weeks after pilocarpine induced SE (or saline treatment).

Discussion

Previously we and others described a decreased GS expression in the hippocampus of TLE patients.[126,127] These data prompted us to investigate GS as a putative peripheral marker for epilepsy. In a group of epileptic children between 4 and 10 years of age, which had not been treated with AEDs, we observed a decrease in leukocyte GS mRNA expression (Bos *et al*. chapter 4). In the present study we investigated

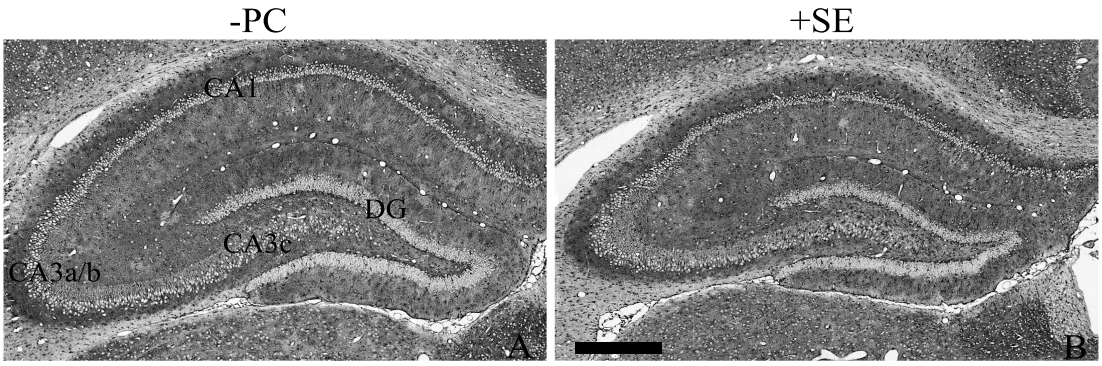

Figure 2. *GS immunoreactivity 2 weeks after pilocarpine induced SE in the hippocampus of an animal with SE (+SE) and of an animal only receiving Li (–PC). CA = cornu Ammonis; DG = dentate gyrus. Scale bar = 500 μm.*

leukocyte GS mRNA expression by qPCR in the juvenile pilocarpine model. GS mRNA expression in leukocytes of pilocarpine treated animals did not differ from that in control animals 2, 4, 8 and 19 weeks after SE induction.

This finding shows that GS mRNA expression is not a marker for epilepsy in this animal model. A key question is whether the juvenile pilocarpine model is suitable to study blood brain correlations in GS expression. In fact, we did not find alterations in GS expression in the hippocampus of these rats 2 weeks after pilocarpine induced SE. Although we did not yet measure GS expression at 4, 8 and 19 weeks after induction of SE, it might be that in this model, hippocampal GS expression is unaffected. This may be due to the fact that we used a mild model of epilepsy in which neuronal degeneration and mossy fiber sprouting are absent (Bos *et al.*, chapter 5). It could also be that hippocampal and leukocyte GS expression changes only occur after a longer period of spontaneous recurrent seizures than at 19 weeks after SE. Thus, it will be interesting to study GS expression in a more severe animal model for TLE and later in the chronic phase after many seizures. However, it could be that even in these models GS is not a marker, because the epilepsy is induced in a laboratory rat strain, which lacks genetic variability and thus the genetic predisposition, which plays an important role in human TLE.[281] In fact, the reduction in GS expression found in children with epilepsy may be a genetic factor, and not a consequence of the epilepsy.

Interestingly, the only change in leukocytes GS mRNA expression we found in animals 2 weeks after treatment in both the –PC and the +SE group. Both groups are pretreated with lithium. Preliminary data in SHAM treated animals show normal GS expression, indicating that lithium induces the transient increase in GS expression. However, at this stage we cannot rule out that the transient increase in GS expression is age-related.

We conclude that GS is not a suitable peripheral marker for epilepsy in the juvenile rat model. Further studies in animal models for TLE with hippocampal sclerosis are required to analyze leukocyte GS expression. It will also be very interesting to determine basal leukocyte GS expression levels in various rat or mouse strains with different susceptibilities to epilepsy, such as the B6 and A/J mouse strains[282] and the chromosome substitution panels derived from these parental strains.[283]

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7 Summary and Discussion

Summary of Results

This thesis describes the research that was undertaken to find peripheral markers for epilepsy and ALS. Changes in the glutamatergic system and excitotoxicity are suggested to play a role in the pathogenesis of epilepsy and ALS *(chapter 1)* and therefore I have focused on those proteins that are important in maintaining low extracellular glutamate concentrations: glutamate transporters (EAATs) and the enzyme glutamine synthetase (GS). In the past, both in ALS and in epilepsy researchers have attempted to find peripheral markers *(chapter 1)* and both plasma and various blood cells have been used, but current knowledge provides us with new tools (e.g. new techniques in molecular biology) and new starting points (cloned transporters and receptors).

As a first step, glutamate transport in human blood platelets was studied *(chapter 2)*. Platelets take-up glutamate in a Na^+-dependent manner, suggesting the involvement of EAATs. EAAT2 protein, but not EAAT1 and EAAT3 protein, was found in human blood platelets. It is likely that EAAT2 contributes to glutamate uptake in resting human platelets, but as basal uptake can be inhibited only by high concentrations of DHK, possibly other transporters are involved as well. On the other hand, it was shown that thrombin activation of blood platelets increased glutamate uptake 9-fold and uptake was DHK sensitive. Indeed kinetic studies suggest a large increase in active transporters, a high level of EAAT2 protein was present on α-granules and thrombin activation leads to α-granule secretion. Thus, the recruitment of EAAT2 from α-granules is most likely the cause of the increase in glutamate uptake.

ALS is an adult onset neurodegenerative disease in which EAAT2 and excitotoxicity have been implicated in the pathogenesis. In contrast to earlier published data,[177] platelets of ALS patients had a normal basal and thrombin-stimulated glutamate uptake and a normal expression of EAAT2 *(chapter 3)*. However, an increased expression of GS protein was demonstrated in ALS patients' platelets. Thus, GS expression in platelets may provide a blood marker for ALS and for the effectiveness of therapy.

As platelets are cell fragments and have no active transcription and translation, we examined the expression of glutamatergic transcripts in leukocytes *(chapter 4)*. Microarray analysis showed that 34 of 52 glutamatergic transcripts were not abundantly expressed on leukocytes, transcripts of 6 genes were more common and 12 transcripts were considered absent. RT-PCR analysis confirmed low amounts of EAAT1, 2 and 3 mRNA in leukocytes, but protein could not be demonstrated. On the other hand, GS mRNA, protein and enzyme activity could be demonstrated in leukocytes. Comparing the expression profile of leukocytes from newly diagnosed epilepsy children with age-matched controls, GS mRNA was approximately 50% decreased. This was confirmed by quantitative PCR (qPCR). After 1.5 years, leukocyte GS mRNA expression was reanalyzed in the same patients and remained lower than in control subjects. As most patients were treated with AEDs, GS expression appeared not to be affected by AED treatment. Thus, leukocyte GS expression seems to be an early marker of epilepsy.

We also examined the leukocytes expression of GTRAP3-18, a protein associated

with EAAT3. The level of GTRAP3-18 mRNA transcripts was slightly (25%) increased in the second measurement (after 1.5 years), but not directly after the diagnosis (*chapter 5*). This might be due to the AED treatment, but as it is measured in a small and heterogeneous group, using different kinds of AEDs, it is difficult to speculate upon the reason or consequences of this increase.
In TLE changes have been found in EAAT1, 2 and 3 and the enzyme GS in hippocampal specimens resected during epilepsy surgery. To study the possible correlation between blood and brain parameters in TLE, we studied EAAT expression in the hippocampus of animals with experimentally induced TLE, using the juvenile pilocarpine model (*chapter 5*). We found a number of distinctly stained cells in the hilus that were intensely stained by antibodies to GLAST and GLT1. The protein expression of GLAST and GLT1 was increased in these animals in the stratum lacunosum-moleculare. These changes in GLAST and GLT1 protein expression were found in animals 2 weeks after the induction of SE and in animals that did not fully developed SE 4 weeks after pilocarpine treatment. Secondly GLAST protein expression was slightly increased in the whole brain of animals 2 weeks after SE. We demonstrated neither a change in EAAC1 expression nor a difference in mRNA transcripts of GLAST, GLT1 and EAAC1. GS protein expression was only examined 2 weeks after SE and was not different from controls. Vimentin staining, a marker for reactive gliosis, was increased at 2 weeks after SE in the stratum lacunosum-moleculare and in the hilus of the DG, suggesting that the changes in GLAST and GLT1 are related to the same process *(chapter 5)*. These changes might reflect temporary changes in glutamatergic transmission and might be part of the mechanism leading to epilepsy.
Using the same animals, we also studied leukocyte mRNA expression with qPCR (*chapter 6*). EAAT1 and EAAT3 transcripts were present in rat leukocytes but were expressed at a level too low for qPCR analysis. GS transcripts were abundantly present in rat leukocytes. QPCR showed no difference in animals after SE, at all time-points tested. Thus, GS seems not to be a peripheral marker of the epileptogenesis in animals of the juvenile pilocarpine model.

General discussion

Research described in this thesis is aimed to identify blood cell markers for neurological diseases associated with glutamate excitotoxicity, such as epilepsy and ALS. The main findings in epilepsy and ALS patients are (1) increased GS protein and (2) normal glutamate uptake in blood platelets of ALS patients, (3) decreased leukocyte GS mRNA expression in epileptic children with and without AED treatment and (4) slightly increased leukocyte GTRAP3-18 mRNA level in children 1.5 years after diagnosis of epilepsy.

Platelet glutamate uptake

In brain tissue, changes in glutamate uptake are found in epilepsy as well as in ALS. Post-mortem brain tissue (spinal cord, motor cortex) of ALS patients showed a major

loss of glutamate uptake, accompanied by a marked decrease in EAAT2 protein expression.[40-42,44] Decreased glutamate transport[51] accompanied by a decreased EAAT2 expression[52] was also shown in the spinal cord of the SOD1 mutant mice model of ALS, but only in the end-stage of the disease.[53,54] In hippocampi resected from epilepsy patients, significant changes in the expression of EAATs have been found[124,125] as well as a decreased synaptosomal glutamate uptake.[284] Adult rats subjected to pilocarpine induced SE showed no change in glutamate uptake 12 hours after the induction of SE[256] but changed expression of glutamate transporters has been shown in different models of epilepsy.[257,258,273-276,285-287]

In blood platelets of ALS patients, we did not detect changes in glutamate uptake under basal conditions or after platelet activation. We showed that glutamate transport in activated platelets was predominantly mediated by EAAT2. EAAT2 protein level in platelet homogenates from these ALS patients was not different from healthy subjects. Thus, this confirmed the findings of a normal glutamate uptake. Our study is at variance with the finding of Ferrarese *et al*. (2001)[177] who showed a 45% decrease in (basal) platelet glutamate uptake in ALS patients. It is difficult to explain the discrepancy between the result of our study in ALS and those of Ferrarese *et al.*, (2001).[177] Most likely, they are related to technical differences between both studies. This is reflected in the different kinetic constants that were determined; K_m and V_{max}. Our study determined kinetic constants similar to the original paper by Mangano and Schwarzc (1981),[157] whereas values determined by Ferrarese *et al*. (2001, 2003)[177,183] are much higher. This may not be too surprising, because we used freshly isolated platelets, whereas Ferrarese et al. stored their platelet samples in the freezer before uptake analysis.

Platelet glutamate transport is also studied in a number of other neurological diseases. In Huntington's disease a normal (basal) platelet glutamate uptake was found,[203] whereas in Alzheimer's disease a reduction of uptake by 40%[182] and a decreased EAAT1 protein expression was found.[183] In Parkinson's disease, a 50% decrease in (basal) platelet glutamate uptake was reported,[181] which correlated with the severity of the disease.[205] Platelet glutamate uptake is also studied in TLE patients with hippocampal sclerosis and although maximal uptake (V_{max}) was not changed, the uptake velocity at low glutamate concentrations was increased.[153] In patients with JME no change in basal glutamate uptake was found.[153] The latter study also investigated short-term *in vitro* effects of the AEDs carbamazepine, lamotrigine and valproate, which did not affected platelet glutamate uptake.[153] This is similar to our observations (unpublished results).

Taken together, although changes found in platelet glutamate uptake might reflect changed occurring in brain tissue in epilepsy and/ or ALS, platelet glutamate uptake does not seem to be a suitable peripheral marker. On the one hand because we could not reproduce the findings of Ferrarese *et al.*[177] in ALS patients, on the other hand because platelet glutamate uptake seems to lack disease specificity, because patients with Alzheimer's disease, Parkinson's disease or ALS all have a 40-50% reduction in platelet glutamate-uptake.

Platelet GS expression

In this thesis work we found an increase in platelet GS protein expression. In temporal lobe epilepsy patients a decrease in GS expression has been reported in hippocampal areas with severe neuron loss.[126,127] In animal models of epilepsy a reduced brain expression is shown as well[277-279] However, GS expression in brain and spinal cord of ALS patients has not been studied so far. On the other hand, extracts of the lower spinal cord of the SOD1 mice, the mutant mice model of ALS, did not show any differences in GS expression or enzyme activity in any stage of the disease compared to unaffected littermates.[201] To investigate if the increase of platelet GS protein is indeed related to changes in brain GS protein, obviously the investigation of GS expression and enzyme activity in ALS post-mortem brain tissue is the first experiment to be done. Secondly, as this is the first study of GS expression blood platelets related to ALS, disease specificity and sensitivity need to be established. It is likely that the expression level of a protein, such as GS, in whole platelet homogenates is a more reliable marker, as opposed to glutamate uptake, because it is not dependent on the activation state of platelets, and does not have to be performed within 6 hours after blood collection.

Leukocytes GS mRNA expression

In epilepsy, this is the first study of GS expression in blood cells. In a small group of children a decreased level of leukocyte GS transcripts is found, which is persistent in all children after 1.5 years, independent of AED treatment. Resected hippocampi from TLE patient with hippocampal sclerosis showed pronounced loss of GS protein in CA1, CA3, CA4 and dentate hilus, areas with severe neuron loss.[126,127] Animal studies show a reduced brain GS expression,[277-279] induction of seizures by inhibiting GS activity[246] and a reduction of brain GS activity after antiepileptic drug treatment.[280] It is unknown if brain GS is also decreased in brain tissue of our patient group, as (obviously) brain tissue of these patients is not examined. If the decrease in leukocyte GS expression reflects a decrease in GS expression in the brain, e.g. due to genetic predisposition, then also a study to the genetic background in epilepsy patients is indicated. The chromosome locus of the GS gene is 1q31 and to data no genetic linkage to epilepsy is found in this region.[288] A number of single nucleotide polymorphism are present in the gene, but further research is required. As this was a first study and we examined a small group of patients, sensitivity and specificity are not yet determined. In conclusion, leukocyte GS expression is a putative marker, but the experiments performed in this reseach are preliminary.

As a first step to learn more about GS as peripheral marker of epilepsy, we wanted to correlate longitudinally brain and blood expression of GS. As longitudinal correlation of brain and blood expression is not feasible in humans, we used the juvenile pilocarpine model. Although rat leukocytes express EAAT1 and EAAT3 mRNA, it was too low abundant to be measured by qPCR. In consequence, we were unable to determine paralleled changes in EAAT expression leukocytes, in relation to the changes observed in the hippocampus of animals 2 weeks after pilocarpine induced SE. We were able to quantify leukocyte GS mRNA, but we found no differences in its mRNA and protein expression level in animals of any of the time-points tested.

Although hippocampal GS expression is not yet tested in the chronic phase of the model, when animals suffer from recurrent seizures, it is clear that GS expression in leukocytes can not serve as a marker for epilepsy in this model. As this is an aquired model of epilepsy, one of the expanations that GS is not a marker in this model, might be that the change in GSis due to genetic variation, which is not present in this laboratory rat strain. Dertermination of the basal leukocyte and brain GS mRNA expression levels in mouse and rat strains with different susceptibility to epilepsy, such as the B6 versus A/J mouse strains[282] might provide answers.

Leukocyte GTRAP3-18 mRNA expression

In this study we found a small increase in leukocyte GTRAP3-18 expression in a small group of children 1.5 years after their diagnosis of epilepsy. Most of these children used AEDs and were seizure-free. Leukocyte GTRAP3-18 transcript level in the one patient not treated with drugs or in the patient treated with AEDs, but was not seizure-free was in the same range as determined in the other patients.

Not much is known about GTRAP3-18. It was found recently in a yeast-two hybrid screen and it interacts with the C-terminal domain of rat homologue of EAAT3.[240] Sequence analysis suggest that GTRAP3-18 the rat homologue of JWA (human vitamin A responsive gene). GTRAP3-18 is expressed widely in the brain and primarily localized to neurons and is localized in the cytoplasm as well as associated to the cell membrane[240] and cytoskeleton.[289] *In vitro* and *in vivo* analysis shows that GTRAP3-18 modulates EAAC1 transport activity by decreasing its affinity for glutamate, but does not influence EAAC1 protein expression and membrane trafficking. Sequence analysis of the protein also suggest the presence of putative serine and tyrosine phosphorylation sites, suggesting the responsiveness to intracellular signaling molecules.[247] In mice, a orthologue is also cloned,[247] and it is shown to be expressed in every tissue tested; brain, heart, liver, kidney and skeletal muscle.[247]

There may be a relationship between an increased GTRPA3-18 expression and epilepsy. Functional deletion of EAAC1 in the rat by antisense oligoneuclotide administration interferes with GABA-synthesis[290] and results in epilepsy and some motor impairment.[3] (EAAT3 knockout mice, however, do not develop spontaneous seizures, but show reduced spontaneous locomotor activity as well as renal abnormalities (dicarboxylic aminoaciduria).[291]) An increase in expression of GTRAP3-18 *in vivo* and *in vitro* resulted in a decreased glutamate uptake, whereas treatment with GTRAP3-18 antisense oligomers resulted in a reduced GTRAP3-18 protein and a significant increase in glutamate uptake. An acquired increase in GTRAP3-18 in brain might result in a decreased uptake, and, as is shown in the EAAC1 antisense treated rats, impaired GABA synthesis[290] and may lead to increased excitability and epilepsy. In mice, GTRAP3-18 is located chromosome region 6D3 synthenic to human chromosome 3p14, where also the human orthologue of GTRAP3-18 is located. Interestingly, linkage was reported for the 3p14-21 locus and idiopathic generalized epilepsy,[292] but was not confirmed in subsequent studies.[293]

Thus, the finding of increased leukocyte GTRAP3-18 mRNA might be a consequence of the epileptic disorder. However, the expression is increased by only 25%, thus a

large functional effect is not suggested. Expression levels in newly diagnosed children with epilepsy and after 1.5 years overlap, making discrimination difficult. The experimental group is still small and heterogeneous with respect to seizure types and AED treatment. A larger study should be performed to confirm the finding. Of course, it would be interesting to examine the expression of GTRAP3-18 in hippocampal tissue of TLE patient with and without hippocampal sclerosis.

Future studies on blood markers

Leukocyte GTRAP3-18 and GS expression in epilepsy

To further investigate the potential of GS and GTRAP3-18 as peripheral markers for epilepsy a new series of experiments is recommended, in larger group of patients; a replication of this study, with newly diagnosed children with epilepsy, carefully classified for seizure type and the influence of AED-treatment should be investigated as well. Another patient group that should be examined are adult TLE patients, preferably just before resection of the hippocampus. In this patient group, brain pathology and expression changes in specific proteins in the resected hippocampus, can be studied simultaneously with blood. In both studies, healthy subjects as well as subjects with other neurological diseases should be included. Although the human studies can give useful answers, longitudinal changes in the brain during epileptogenesis can only be studied in animal models. Therefore the investigation of other epilepsy models is indicated.

Of course peripheral markers have the disadvantage that peripheral variables might also influence the parameters. Moreover, the function of these proteins in peripheral tissues is sometimes different from the function in brain. When studying blood or blood cells, this should be kept in mind. For instance, the feeding pattern can influence the composition of blood and infections cause changes in the composition of subsets of leukocytes in blood. Also, the effect of treatment of the disease must be discerned from the direct effects of the drugs on blood cells. For instance, a number of drugs distort normal thrombosis (e.g. valproate[165]) and/or might cause thrombocytopenia or leucopenia.[294-296] Therefore, the direct effects of different drug should be taken into account in further research.

New directions

Early markers can be of great importance especially in neurological diseases like epilepsy, where a latent period is observed between the clinical seizures and the precipitating injury, or in ALS, where clinical signs are first seen, when a significant number of neurons is already degenerated. An early start of treatment to halt or even reverse pathogenic mechanisms (if available) is in those diseases essential and the research to these markers is of great importance.

Research described in this thesis is focused on proteins involved in the glutamate-glutamine cycle. Although we found changes in GS expression in blood samples the

number of genes we studied was very limited. A new approach can been found in microarray studies, where the expression pattern of up to 30,000 genes can be studies simultaneously in mRNA isolated from whole blood or leukocytes. This method is first published in 1995 by Schena et al.[297] and is becoming increasingly available in the last 3-4 years. It has proven to be a sensitive method to develop and refine the molecular taxonomy of cancer and other disorders, as well as a tool to investigate molecular heterogeneity.[241,298]

In a number of neurological diseases microarray technology has been used to identify unique expression patterns associated with the disease both in brain tissue as well as in blood. In autoimmune diseases, including multiple sclerosis, to date the most detailed analysis of blood expression patterns has been carried out.[299-301]

Tang *et al*.[218,219] determined whether different epilepsy patient groups were associated with unique patterns of up- and down-regulated genes in whole blood. Patients, children with epilepsy, treated with carbamazepine or valproate showed significantly different expression patterns than untreated patients. Moreover, valproate responders could be distinguished from valproate-resistant patients, among others because many mitochondrial genes were overexpressed in valproate responders. Unfortunately, they did not include healthy control children in this study and details of specific genes that are up- or down-regulated are not revealed in these papers.[218,219] Thus, this shows that microarray studies also may provide a powerful tool to determine effectiveness of drug treatment or even help defining individual patient's pharmaco-therapeutic profiles. Such profiles could contribute to the choice of pharmacotherapy. This is particularly relevant in epilepsy, where the effectiveness of AEDs varies considerably between patients.

In *chapter 4*, we also performed microanalysis of leukocyte gene expression profiles in a small number of newly diagnosed epileptic children. In line with the aim of this thesis we focused on glutamatergic genes, but further analysis of the experimental data is possible and underway, as well as the microarray analysis of the expression levels in the leukocyte samples taken 1.5 years later.

In conclusion, the new technology of microarray analysis can provide valuable tools to determine new peripheral markers or marker sets of neurological diseases, including epilepsy, which can be used in early diagnosis. Simultaneous determining gene expression of a large number of genes might reveal specific patterns associated with specific seizure of epileptic disorder subtype, or can be used in prediction or monitoring the effectiveness of a certain drug. However, of highest importance is the careful longitudinal analysis of patients and controls, regarding disease pathology, clinical phenotype, drug treatments, immunologic markers and the use of strict inclusion and exclusion criteria.

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Samenvatting

Dit proefschrift beschrijft het onderzoek dat gedaan is om perifere bloedmarkers voor de hersenziekten epilepsie and ALS te vinden. Een perifere marker zou kunnen helpen bij het vroegtijdig stellen van een diagnose, het indelen van patiëntengroepen, het voorspellen of bepalen van de werkzaamheid van medicatie.

Epilepsie, ook wel 'de vallende ziekte' genoemd, is een ziekte die zich kenmerkt doordat de patiënten stuipen of aanvallen hebben. Epilepsie is een verzameling van syndromen met aanvallen als symptoom. De incidentie is 1 op 150. Een vorm van epilepsie is temporaal kwab epilepsie (TLE), hierin beginnen de aanvallen in de temporaalkwab (slaapkwab) van de hersenen. De meeste patiënten met TLE kunnen goed behandeld worden met medicijnen, maar ongeveer 25% van de patiënten wordt resistent tegen anti-epileptica. In deze patiënten kan de epileptogene zone (het gebied waar de aanvallen ontstaan) chirurgische worden verwijderd. Meestal wordt een deel van de temporaalkwab en de hippocampus uitgenomen. Omdat dit weefsel beschikbaar is voor wetenschappelijk onderzoek is er inmiddels relatief veel kennis over deze vorm van epilepsie (*hoofdstuk 1*).
Amyotrofe lateraal sclerose (ALS) is een zeldzame ziekte waarbij vooral neuronen afsterven, die de beweging van de spieren aansturen. Dit resulteert in progressieve spierzwakte en spier afsterving. Deze ziekte heeft een veel lagere incidentie met ongeveer 150 patiënten in Nederland. De ziekte heeft een progressief verloop en is ongeneselijk (*hoofdstuk 1*).
In zowel epilepsie als ALS lijkt excitotoxiciteit door de neurotransmitter glutamaat een rol te spelen in de pathogenese. Dit is de reden dat het onderzoek naar perifere markers gefocust is op de eiwitten die belangrijk zijn voor het bewaken van de lage extracellulaire glutamaat concentraties in het brein. Dit zijn vooral de glutamaat transporter eiwitten (EAAT1-5) die zorgen voor het wegpompen van het extracellulaire glutamaat en het enzym glutamine synthetase (GS) dat zorgt voor de omzetting van glutamaat in glutamine (*hoofdstuk 1*).
Als eerste werd glutamaat-opname in bloedplaatjes onderzocht, afkomstig van gezonde controle personen. Glutamaat-opname in bloedplaatjes was Na^+-afhankelijk. Het bleek dat EAAT2 eiwit kon worden aangetoond in bloedplaatjes, maar EAAT1 en EAAT3 niet. De basale glutamaat-opname kon worden geremd door algemene remmers van glutamaat-transport, maar niet door een EAAT2 specifieke remmer. Dit suggereert dat EAAT2 een bijdrage levert aan de glutamaat opname onder basale condities, maar de aanwezigheid van andere EAATs kon niet worden uitgesloten. Thrombine, een stof die in bloed betrokken is bij de bloedstolling en in dit proces verantwoordelijk is voor de activatie van bloedplaatjes, zorgde voor een sterke toename van de Na^+-afhankelijke glutamaat-opname. Deze toename kon worden geremd door de EAAT2 specifieke remmer dihydrokainate. Een combinatie van deze inhibitor-studie, kinetische analyse en eiwitbepaling in verschillende subcellulaire fracties van bloedplaatjes toonde aan dat de verhoging van glutamaat-opname na thrombine activatie van bloedplaatjes waarschijnlijk EAAT2 gemedieerd is en veroorzaakt wordt doordat er nieuwe glutamaat transporters (EAAT2) vanuit de α-granula in de buitenmembraan worden ingebouwd (*hoofdstuk 2*).
In ALS patiënten werd vervolgens onderzocht of de glutamaat-opname in bloedplaatjes en de hoeveelheid EAAT2 anders was dan in gezonde controle

personen. In tegenstelling tot andere onderzoekers vonden we dat ALS patiënten een normale basale en thrombine-gestimuleerde, Na^+-afhankelijke glutamaat-opname hebben in hun bloedplaatjes. De hoeveelheid EAAT2 eiwit in bloedplaatjes van ALS patiënten was ook normaal, maar de hoeveelheid van het enzym GS bleek in ALS patiënten significant verhoogd te zijn in vergelijking met gezonde controles. Het is mogelijk dat de expressie van GS in bloedplaatjes een perifere marker van ALS is, maar meer onderzoek hiernaar is nodig (*hoofdstuk 3*).

Omdat bloedplaatjes celfragmenten zijn, waarin aanmaak van nieuwe eiwitten niet plaats vindt, hebben we vervolgens de genexpressie in leukocyten (witte bloedcellen) onderzocht. Met microarrayanalyse hebben we aangetoond dat leukocyten een aantal genen tot expressie brengen die betrokken zijn bij de glutamaterge signaal transductie. De vergelijking van de leukocyten-expressie van deze genen tussen pas gediagnosticeerde epilepsie patiëntjes en controles toonde aan dat de GS expressie verlaagd was met 50% in epilepsie patiëntjes. Deze bevinding werd bevestigd met een andere techniek, kwantitatieve PCR (qPCR). Dit verschil was ook 1,5 jaar later nog aanwezig, ondanks het gebruik van verschillende anti-epileptica. In de literatuur is beschreven dat de GS expressie verlaagd is in de hippocampus van HS-TLE patiënten. Genetische predispositie voor epilepsie door genetische aanleg voor lagere GS expressie zou een oorzaak kunnen zijn voor het vinden van zowel een afname in de hippocampus als in de leukocyten van deze patiënten. Hoewel de data veelbelovend zijn, dient de geschiktheid van GS expressie in leukocyten als marker voor epilepsie verder te worden onderzocht; bijvoorbeeld wat betreft de sensitiviteit en selectiviteit, de invloed van aanvalstype en/of medicijngebruik (*hoofdstuk 4*).

We onderzochten ook de aanwezigheid van GTRAP3-18, een eiwit geassocieerd met EAAT3 in deze epilepsie patiënten. Genexpressie in leukocyten van GTRAP3-18 bleek met 25% toegenomen in epilepsie patiënten na 1,5 jaar behandeling. Dit zou kunnen komen door gebruik van medicatie, maar vervolgonderzoek is nodig om de relatie van deze toename met bijvoorbeeld de duur van de epilepsie, het aanvalstype en het gebruik van medicatie te achterhalen (*hoofdstuk 4*).

Om de mogelijke relatie tussen gevonden veranderingen in de hippocampus en bloed in TLE te onderzoeken, hebben we de expressie van EAATs onderzocht in een diermodel voor TLE, het juveniele pilocarpine model. In de hippocampus van ratten, 2 weken na behandeling, maar niet op de latere tijdstippen, werd een algemene verhoging van GLAST (EAAT1) kleuring waargenomen, de GLAST en GLT1 (EAAT2) kleuring in de stratum lacunosum-moleculare in de CA-gebieden was verhoogd en in de hilus van de DG (CA3c) waren een aantal cellen sterk aangekleurd voor GLAST en GLT1. Precies in die gebieden werd een verhoging van de vimentine immunoreactiviteit gevonden. Dit duidt op aanwezigheid van reactieve astrocyten. GS expressie was niet veranderd 2 weken na behandeling, maar is op de latere tijdstippen (nog) niet onderzocht. Ook EAAC1 (EAAT3) was niet veranderd. Mogelijk duidt de verhoging in GLAST en GLT1 op een verhoogde glutamaat signaaltransductie en is er een verband met de activatie van astrocyten en de epileptogenese die plaatsvindt in deze dieren (*hoofdstuk 5*).

Van dezelfde dieren werd ook bloed afgenomen om leukocyten mRNA expressie met qPCR te kunnen onderzoeken. EAAT1 en EAAT3 transcripts waren wel aanwezig op leukocyten van ratten, maar in een te lage hoeveelheid om geschikt te zijn voor qPCR

analyse. GS mRNA in leukocyten kon wel worden geanalyseerd en bleek niet veranderd in dieren die behandeld waren met lithium-pilocarpine, op alle vier geteste tijdpunten. Hoewel GS expressie in de hippocampus van deze dieren nog niet bepaald is in de chronische fase, lijkt GS mRNA in leukocyten geen perifere marker te zijn voor epileptogenese in wistar ratten uit het juveniele pilocarpine model (*hoofdstuk 6*).
Samenvattend werd geconcludeerd dat glutamaat-opname in bloedplaatjes geen geschikte perifere marker is voor ALS. In de eerste plaats omdat wij, in tegenstelling tot anderen, geen veranderde glutamaat-opname vonden in ALS patiënten, waardoor het blijkbaar geen betrouwbare marker is. Bovendien is in meerdere neurologische ziekten een afname gevonden in glutamaat-opname in bloedplaatjes en lijkt het geen specifieke marker te zijn. De bepaling van totaal GS eiwit in bloedplaatjes lijkt een meer betrouwbare marker voor ALS. De relatie met brein GS in ALS is (nog) niet vastgesteld en is een voor de hand liggend vervolgonderzoek. In epilepsie is zowel in de hippocampus van HS-TLE patiënten als ook in leukocyten van kinderen met epilepsie gevonden dat de GS expressie verlaagd is. Genetische aanleg zou hierbij een rol kunnen spelen. Deze marker moet nader onderzocht worden met nadruk op de specificiteit, sensitiviteit, gevoeligheid voor behandeling met AEDs en de directe invloed van perifere processen (*hoofdstuk 7*). Microarrayanalyse van genexpressie in leukocyten lijkt een succesvolle aanvullende methode om perifere markers te onderzoeken en is een methode die in toekomstige experimenten nader uitgewerkt moet worden. Expressie profielen van leukocyten kunnen een waardevolle aanvulling zijn bij bijvoorbeeld de diagnose en de classificatie van epilepsie (*hoofdstuk 7*).

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Curriculum Vitae

Willemina Minke (Ineke) Bos werd op 4 september 1975 geboren in Groningen. In 1992 behaalde zij haar HAVO diploma aan het het Wessel Gansfort College te Groningen.
Het volgende jaar ging zij Biotechnologie studeren aan de Noordelijke Hogeschool Leeuwarden. De eerste stage voor deze studie deed zij bij de vakgroep Biologische Psychiatrie onder leiding van Dr. G.J. ter Horst. Tijdens deze stage onderzocht zij de centrale verwerking van subcutane en cardiale pijn in de rat. Haar tweede stage deed zij bij Prof. Dr. R. Schmitt, Lehrstul Genetik, Universität Regensburg te Regensburg, Duitsland. Hier heeft zij drie genen betrokken bij de flagelrotatie van de bacterie *R.meliloti* bestudeerd. In 1997 heeft zij deze HBO-opleiding met als afstudeerrichting Medische en Dierlijke Biotechnologie afgesloten.
In datzelfde jaar ging zij Biologie studeren aan de Rijksuniversiteit Groningen. Haar eerste afstudeerstage deed zij wederom bij Dr. G.J. ter Horst, vakgroep Biologische Psychiatrie, met nu als onderwerp de invloed van de thalamus op pijn-gedrag in de rat. Bij de vakgroep Histologie en Celbiologie deed zij onderzoek naar *in vivo* en *in vitro* T-cell reactie op infectie met RCMV in diabetis gevoelige ratten onder leiding van Dr. JL. Hillebrands. In mei 2000 behaalde zij het doctoraal diploma Biologie, specialisatie Medische biologie.
Op 1 januari 2001 begon zij als assistent in opleiding bij het Rudolf Magnus Instituut een onderzoek naar perifere markers van epilepsie onder begeleiding van Prof. Dr. O. van Nieuwenhuizen, Prof. Dr. J.P.H. Burbach en Dr. P.N.E. de Graan. De resultaten van dit onderzoek zijn beschreven in dit proefschrift.

Affilation of (co)authors

Ineke WM Bos, W Saskia van der Hel, Koen L.I. van Gassen, Pierre NE de Graan, Caroline Meine Jansen, Marije G. Rensen-de Leeu,w Henk Spierenburg, Elly J.F. Vereyken, Gineke Wassink, Marina de Wit.
Rudolf Magnus Institute of Neuroscience, Department of Pharmacology and Anatomy, University Medical Center Utrecht, Utrecht, The Netherlands.

Onno van Nieuwenhuizen
Rudolf Magnus Institute of Neuroscience, Department of Child Neurology, University Medical Center Utrecht, Utrecht, The Netherlands.

Leonard H van de Berg
Rudolf Magnus Institute of Neuroscience, Department of Neurology, University Medical Center Utrecht, Utrecht, The Netherlands.

Gijsbert van Willigen
Department of Haematology, University Medical Center Utrecht, Utrecht, The Netherlands.

Govert Hoogland
Department of Neurosurgery, Academic Hospital Maastricht, Maastricht, The Netherlands.