SIRPA RAINESALO

GABA and Glutamate in Human Epilepsies

Studies on the Platelet Model

■

ACADEMIC DISSERTATION

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ABBREVIATIONS

AED	antiepileptic drug
ALS	amyotrophic lateral sclerosis
AMPA	2-amino-3-hydroxy-5-methyl-4-isoxazolepropionate
BBB	blood-brain barrier
BGT-1	betaine/GABA transporter
BZP	benzodiazepine
CAE	childhood absence epilepsy
cAMP	cyclic adenosine monophosphate
CBZ	carbamazepine
cDNA	complementary DNA
CNS	central nervous system
CPS	complex partial seizure
CSF	cerebrospinal fluid
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EAAC	excitatory amino acid carrier
EAAT	excitatory amino acid transporter
EDTA	ethylenediaminetetra-acetate
EEG	electroencephalogram
ESM	ethosuximide
GABA	γ-aminobutyrate
GAD	glutamate decarboxylase
GAERS	genetic absence epilepsy in rats from Strasbourg
GAT	GABA transporter
GBP	gabapentin
GLAST	glutamate-aspartate transporter
GLT	glutamate transporter
GTCS	generalized tonic-clonic seizure
HS	hippocampal sclerosis
IGE	idiopathic generalized epilepsy
ILAE	International League Against Epilepsy
JME	juvenile myoclonic epilepsy
LTG	lamotrigine
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MTLE	mesial temporal lobe epilepsy
NMDA	N-methyl-D-aspartate
OXC	oxcarbamazepine
PHT	phenytoin

RLE	refractory localization-related epilepsy
RNA	ribonucleic acid
RT-PCR	reverse transcription-polymerase chain reaction
S.D.	standard deviation
SDS	sodium dodecylsulfate
SEM	standard error mean
SPS	simple partial seizure
TLE	temporal lobe epilepsy
TLE+HS	temporal lobe epilepsy with hippocampal sclerosis
TPM	topiramate
VPA	valproate
ZON	zomisamide

ABSTRACT

Epilepsy and seizures have been proposed to be associated with an imbalance in the levels of inhibitory and excitatory amino acids. The main inhibitory amino acid transmitter in the human central nervous system (CNS) is γ-aminobutyrate (GABA) and the major excitatory amino acid transmitter glutamate. The actions of GABA and glutamate in the CNS are terminated by uptake into neurons and glia, this beeing mediated by transporters for both transmitters. Blood platelets have been used as a model for GABA and glutaminergic neurotransmission. It is presumed that the platelet functions are similar to those in the CNS. Platelets have been used in studies on different neurological diseases, but epilepsy studies on the platelet model are scant.

We measured now the uptake of GABA and glutamate into platelets, the activity of GABA transaminase (GABA-T) activity in platelets and the concentrations of amino acids in plasma and cerebrospinal fluid. Adult patients with juvenile myoclonic epilepsy (JME), refractory localization-related epilepsy (RLE), temporal lobe epilepsy and hippocampal sclerosis (TLE+HS) and healthy controls were involved in the studies. We also assessed the plasma amino acid concentrations after acute tonic clonic seizures. GABA uptake and GABA-T activity were likewise assessed in pediatric patients with absence epilepsy (CAE).

The capacity of GABA uptake in platelets was decreased in the adult patients with JME and RLE. The activity of GABA-T was altered in those with JME and CAE, being increased and decreased, respectively. The uptake of glutamate was enhanced in patients with TLE+HS when compared to healthy controls, but in JME patients it did not differ from that in controls. When tested in vitro, only ethosuximide (ESM) showed GABA uptake by the platelets to be diminished in a concentration-dependent manner at clinically relevant ESM concentrations. None of the other commonly used antiepileptic drug directly affected these uptake processes or GABA-T activity in platelets in our patient population.

Our results imply marked differences in the functions of GABA, especially in idiopathic epileptic syndromes. The changes in glutamate metabolism, in patients with partial epilepsy, seem to be compensatory to the increased glutamate levels in plasma after seizures. The changes in these transmitters are apparently syndrome-specific.

We also cloned transporters for GABA and glutamate from human blood platelets. The transporters found in the platelets were betaine/GABA (BGT-1) transporter and excitatory amino acid transporter 3 (EAAT3). The cloning of these transporters confirms that blood platelets can be used as a model for the transport of at least some neurotransmitters in the CNS.

INTRODUCTION

Epilepsy is a common neurological disorder characterized by recurrent seizures. Up to 10 % of the general population experience at least one seizure during their lifetime and 1% of the population have manifest epilepsy (Hauser et al. 1996; Sander 2003). Epilepsy has been known since Antiquity and previously involved a strong social stigma due to the bizarre nature of epileptic seizures. Advances in epilepsy research have led to a better understanding of the disease and also to improved treatment of seizures. However, there still remain approximately 30% patients that have refractory epilepsy and continuous seizure activity (Kwan and Brodie 2000).

γ-Aminobutyrate (GABA) is the major inhibitory and glutamate the major excitatory amino acid in the human central nervous system (CNS). The amino acid transmitters GABA, glutamate and aspartate are probably involved in the pathomechanisms of epileptic seizures. Changes in the inhibitory and excitatory amino acid transmitters have hence been thought to be responsible for epilepsy. The concentrations of these amino acids have been shown to be altered during epileptic activity in the human brain, leading to an imbalance between excitation and inhibition (During and Spencer 1993; Wilson et al. 1996). Changes in GABA metabolism, release or transport may be of significance in suppressing the origin and spreading of seizure activity (Schechter et al. 1984). Increased glutamatergic excitation in epilepsy may likewise arise from excessive presynaptic release and/or altered glutamate uptake. Glutamate is thought to have a role in the induction and spreading of seizures as well as in seizure-related neuronal damage (Meldrum 1994).

Human platelets were first used as a model for the transport of serotonine (Paasonen 1968; Lingjaerde 1969; Paasonen 1973) and later for that of amino acids, although it has not been known exactly which transporters operate in platelets. It has been postulated that the functions of platelets are similar to those in the CNS (Zieve and Solomon 1968; Mangano and Schwarcz 1981a; Hambley and Johnston 1985). Earlier studies with platelets have demonstrated changes in the uptake of GABA and glutamate in many neurological diseases such as amyotrophic lateral sclerosis (ALS), Alzheimer's disease, Parkinson's disease and Down's syndrome (Enns and McCoy 1980; Ferrarese et al. 1999, 2000, 2001a; Begni et al. 2003; Zoia et al. 2004).

More information is needed regarding the role of amino acid transmitters in epilepsy. The present study was designed to clarify the possible changes occurring in the functions of GABA and glutamate in epilepsy. The aim was to evaluate changes in different epilepsy syndromes and also to prove the applicability of the platelet model in epilepsy studies.

REVIEW OF THE LITERATURE

1. Epilepsy

1.1. Definition

The epileptic seizure is attributable to an abnormal excessive neuronal activity, resulting in transient disruption in brain functions manifesting as interruption of consciousness and involuntary movements. Seizures are either unprovoked (spontaneous) or provoked. Epilepsy consists in a proneness to the occurrence of unprovoked epileptic seizures. It is a variety of symptoms, having many different pathomechanisms and is one of the most common neurological diseases. Epileptic syndromes are classified using a number of aspects such as the type of seizures, age at onset, family history and findings in electroencephalogram (EEG) and neuroimaging studies. Each epileptic syndrome has its own natural course in terms of history and prognosis.

1.2. Epidemiology and etiology

The incidence of epilepsy is high in early childhood, decreasing in young people, and increasing again in the elderly (Hauser et al. 1993; Halatchev 2000; Kotsopoulos et al. 2002). The prevalence of epilepsy is 4-10/1000 and the incidence 44-72/100 000 in the industrialized countries (Forsgren 1992; Hauser et al. 1993; Forsgren et al. 1996; Bell and Sander 2001; Cowan 2002; Kotsopoulos et al. 2002; Sander 2003). The lifetime prevalence rates are higher than the prevalence of active epilepsy; up to 5 % of people will experience a non-febrile epileptic seizure during their lifetime (Bell and Sander 2001; Sander 2003). Epilepsy is more common in males. In early life, epilepsy is often due to genetic, developmental or congenital factors, whereas later epilepsy may result, for instance, from cerebrovascular diseases, head trauma, infections or tumors (Hauser et al. 1993; Forsgren et al. 1996).

1.3. Classification

The International League against Epilepsy (ILAE) (1989) has published a classification of epilepsies and epileptic syndromes (Table 1). The classification divides epilepsies into two main categories, generalized and localization-related, which are further subdivided into idiopathic and symptomatic or cryptogenic

epilepsies. Idiopathic epilepsies may be inherited and are presumed to result from abnormalities in neurotransmission without any structural changes in the brain. They usually arise in childhood or adolescence. Symptomatic epilepsies are a result of a known structural disease or cause, which can often be seen in imaging studies. Cryptogenic epilepsies are presumed to have a structural cause not amenable to demonstration. ILAE is now in the process of renewing the classification (www.ilae.org).

Juvenile myoclonic epilepsy (JME) and childhood absence epilepsy (CAE) are both categorized as idiopathic generalized epilepsies (IGEs), while temporal lobe epilepsy is included in the localization-related forms.

1.3.1. Juvenile myoclonic epilepsy

Juvenile myoclonic epilepsy (JME) is one of the most important IGEs, belonging to the group of inherited epileptic myoclonic syndromes. It has a favorable outcome, compared for example with another inherited myoclonic epilepsy, progressive myoclonic epilepsy (Leppik 2003). The prevalence of JME is 8-10% amongst adult and adolescent patients with epilepsies. Both sexes are evenly affected (Genton et al. 2000; Panayiotopoulus 2002a). JME appears around puberty. It is characterized by bilateral, single or repetitive, arrhythmic, irregular myoclonic jerks on awakening, generalized tonic-clonic seizures (GTCSs) in nearly all patients and in some cases also infrequent absences (Genton et al. 2000; Panayiotopoulus 2002a). Rapid, generalized polyspikes and waves are seen in the interictal EEG. Five to 20 spikes with a frequency of 12-15 Hz precede the slow wave. The frequency of the slow waves is 3-5 Hz. These kinds of EEG changes have been found in 22-60% of patients with JME. The changes are commonly accentuated by hyperventilation, sleep deprivation or photic stimulation (Waltz 2000). JME responds well to antiepileptic drugs (AEDs), but typically needs life-long treatment. The risk of seizure relapse after discontinuation of drugs approaches 90 % (Schmidt 2000).

JME has a strong genetic background. The cause of its classical forms has been traced to chromosome 6p11 (Delgado-Esqueta et al. 1990), but the model of inheritance is complex and heterogenic (Robinson and Gardiner 2000). Also changes have been reported in genes encoding the $GABA_A$ receptor (Cossette et al. 2002).

1.3.2. Childhood absence epilepsy

Childhood absence epilepsy (CAE) is a syndrome characterized by brief absence seizures in otherwise healthy children with normal intelligence and typical bilateral symmetrical synchronized 3 Hz spikes and wave discharges in EEG (ILAE 1989). CAE accounts for 2-10% of all childhood epilepsies (Avoli et al. 2001). The onset is commonly between 4-8 years, the peak age being 6-7 years. Two thirds of patients are girls. Some patients may also have sporadic tonic-clonic, febrile or myoclonic seizures. Eyelid myoclonic absence epilepsy is a subtype of CAE. Two thirds of patients can be expected to enter long-term remission (Wirrell 2003). CAE is genetically determined. The model of transmission is unknown, but genetic studies have implicated an alteration in the genes encoding $GABA_A$ receptor subunits (Wallace et al. 2001; Marini et al. 2003).

1.3.3. Temporal lobe epilepsy

Temporal lobe epilepsy (TLE) belong to the localization-related epilepsy syndromes, being the most common partial epilepsy in adulthood. Partial seizures account for 50-60% of all epilepsies (Keränen et al. 1988; Wiebe 2000). Approximately 30-60% of them originate in the temporal lobe (Manford et al. 1992; Wiebe 2000). TLE can be subclassified into mesial (MTLE) and lateral or neocortical temporal lobe epilepsies. Two thirds of TLEs are MTLEs and one third lateral or neocortical temporal epilepsies (Panayiotopoulus 2002b).

TLE often begins in early adolescence. Simple partial, complex partial and secondarily generalized seizures or combinations of them characterize the condition. Epigastric aura, fear and oro-alimentary and gestural automatisms and disturbances in consciousness are the most common ictal symptoms. There is frequently a history of febrile seizures and a family history of seizures. Unilateral or bilateral temporal lobe spikes are common in EEG (Panayiotopoulus 2002b).

MTLE is often characterized by hippocampal sclerosis (HS); a unique pattern of cellular loss which is not uniform in the hippocampus, and gliosis. There is neuronal cell loss in the CA1 and CA3 regions (Arroyo et al. 2002). This is the most common pathomechanism in TLE. The hippocampal cell loss leads to a reorganization of remaining neurons which causes abnormal discharges leading to clinical seizures (Babb et al. 1984; Babb 1999). It is a matter of controversy whether or not HS is the cause or the reason in TLE. Magnetic resonance imaging (MRI) studies have shown that the duration of epilepsy and the number of seizures are related to the severity of hippocampal changes (Kälviäinen et al. 1998; Theodore and Gaillard 2002).

Table 1. International classification of epilepsies and epileptic syndromes (Commission on Classification and Terminology, ILAE 1989)

Class	Classification
1.	Localization-related (focal, local, partial) epilepsies and syndromes
1.1	Idiopathic (with age-related onset)
	Benign childhood epilepsy with centrotemporal spikes
	Childhood epilepsy with occipital paroxysm
	Primary reading epilepsy
1.2	Symptomatic
	Chronic progressive epilepsia partialis continua of childhood
1.3	Cryptogenic
2	Generalized epilepsies and syndromes
2.1	Idiopathic (with age-related onset, in order of age)
	Benign neonatal familial convulsions, Benign neonatal convulsions
	Benign myoclonic epilepsy of infancy
	Childhood absence epilepsy (pyknolepsy), Juvenile absence epilepsy
	Juvenile myoclonic epilepsy
	Epilepsy with grand mal seizures on awaking
	Other idiopathic generalized epilepsies not defined above
	Epilepsies with seizure precipitated by specific modes of activation
2.2	Cryptogenic or symptomatic (in order of age)
	West syndrome
	Lennox-Gastaut syndrome
	Epilepsy with myoclonic-astatic seizures
	Epilepsy with myoclonic absences
2.3	Symptomatic
2.3.1	Nonspecific etiology
	Early myoclonic encephalopathy
	Early infantile epileptic encephalopathy with suppression-burst
	Other symptomatic generalized epilepsies not defined above
2.3.2	Specific syndromes (see the original reference)
3	Epilepsies and syndromes undetermined whether focal or generalized
3.1	With both generalized and focal seizures
	Neonatal seizures
	Severe myoclonic epilepsy of infancy
	Epilepsy with continuous spike-waves during sleep
	Acquired epileptic aphasia (Landau-Kleffner syndrome)
	Other undetermined epilepsies not defined above
3.2	Without unequivocal generalized or focal features (e.g. many cases of sleep-grand mal)
4.	Special syndromes
4.1	Situation-related seizures
	Febrile convulsions
	Isolated seizures or isolated status epilepticus
	Seizures due to acute metabolic or toxic factors such as alcohol, drugs, eclampsia

1.4. Outcome of seizures in epilepsy

Although epilepsy is at present mostly well manageable with modern antiepileptic drugs, there still remain approximately 30% of patients with epilepsy which does not respond to adequate AED treatment. They are then said to have refractory epilepsy (Kwan and Brodie 2000). Most patients with refractory epilepsy have partial seizures, most commonly TLE.

Refractory epilepsy is thought to be a progressive disorder but there are no good early markers to identify it. Among possible clues may be the failure of the first AED to have an effect, the duration of treatment without seizure control and the presence of symptomatic or cryptogenic epilepsy (Kwan and Brodie 2000; Lindsten et al. 2001; Arroyo et al. 2002). Also some MRI findings may predict epilepsy to be refractory, for example hippocampal sclerosis (Arroyo et al. 2002). The number of seizures before treatment as a predictor of refractory epilepsy is controversial. Earlier studies by Sillanpää (1993), Musicco and associates (1997) and Kwan and Brodie (2000) failed to show a high number of seizures to increase the risk of future seizures, but Hauser and Lee (2002) recently reported that in some patients this may be the case.

There are also differences between the epilepsy syndromes in the outcome of seizures. West and Lennox-Gastaut syndromes are traditionally thought of as refractory, catastrophic epilepsies (Shneker and Fountain 2003). Likewise, persistent seizures are often a sign of TLE+HS (Arroyo et al. 2002). Changes in neurotransmitter functions may also lead to refractory epilepsy. In TLE patients, the expression of hippocampal GABA and glutamate transporters seems to be altered (Mathern et al. 1999). Changes have been shown in the subunit composition of transmitter receptors (Blumcke et al. 1999). There are also studies on multidrug resistance genes which participate in refractory epilepsy by affecting the penetration of the drugs to the epileptic focus, since the expression of drug transporters at the blood-brain barrier (BBB) exhibits differences (Tishler et al. 1995; Ling 1997; Regesta and Tanganelli 1999; Dombrowski et al. 2001; Marroni et al. 2003; Siddiqui et al. 2003; Sisodiya 2003).

1.5. Antiepileptic medication

Monotherapy is recommended if seizure freedom is achieved in this manner, but there is sometimes a need for polytherapy in epilepsy. Approximately 60% of patients achieve seizure freedom by monotherapy (Kwan and Brodie 2000). If polytherapy is necessary, it should be rational. The mechanisms of action should be considered when combining drugs. Anticonvulsant drugs generally act by (1) enhancing GABAergic mechanisms, (2) impeding excitatory glutamate neurotransmission, (3) depressing voltage-dependent Ca^{2+}-mobilizing mechanisms

or (4) depressing Na^+ conductance (Ure and Perassolo 2000). Many AEDs have mixed mechanisms of action.

Sodium channel blocking is the traditional action mechanism of AEDs. These drugs include phenytoin (PHT), carbamazepine (CBZ), oxcarbazepine (OXC), and lamotrigine (LTG). Some other drugs also exhibit sodium channel-blocking activity in addition to their other effects. Such are valproate (VPA), zonisamide (ZON), gabapentin (GBP) and topiramate (TPM) (Keränen et al. 1997; Morrell 2002; White 2003).

The GABAergic drugs have different mechanisms of action. Vigabatrin (VGB) is a selective GABA-T inhibitor (Keränen et al. 1997), tiagabine (TGB) inhibits GABA uptake (Morrell 2002) and barbiturates and benzodiazepines (BZPs) act at $GABA_A$ receptors (Morrell 2002). Of the newer antiepileptics, TPM and ZON are thought to have an effect on $GABA_A$ receptors in addition to other mechanisms of action (White 2003). GBP elevates the GABA levels in the brain by enhancing GABA synthesis (Czuczwar and Patsalos 2001; White 2003). Also VPA raises the concentration of GABA in the blood after subchronic treatment (Löscher and Schmidt 1980).

TPM is also thought to exert its effect by blocking kainate/2-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors and altering glutamate levels (Morrell 2002; White 2003). Felbamate possesses N-methyl-D-aspartate (NMDA) -antagonistic effects combined with other mechanisms (Meldrum 2000). The drugs which work through calcium channels include ethosuximide (ESM), GBP, LTG, TPM, OXC, ZON and VPA (Morrell 2002; White 2003).

There are also AEDs whose mechanism of action is unknown. Levetiracetam is a novel AED whose precise action mechanism is still unknown. It may block the N-type of calcium channels (Lukyanetz et al. 2002). Though VPA has been used for several decades, its mechanism of action is likewise not yet fully understood.

2. Epilepsy and the concentrations of amino acid transmitters

There is compelling evidence of an imbalance between excitatory and inhibitory neurotransmitters in epilepsy (Emilien and Maloteaux 1998; Olsen et al. 1999). The amino acid transmitters GABA, glutamate and possibly aspartate, and the neuromodulator taurine are involved in the pathomechanisms of epileptic seizures. The concentrations of these amino acids have been shown to change during epileptic activity in the human brain, perturbing the balance between excitation and inhibition (Kish et al. 1988; During and Spencer 1993; Wilson et al. 1996; Labiner et al. 1999).

GABA is the most important inhibitory neurotransmitter in the human CNS. Changes in GABA metabolism may be significant in suppressing the origin and spread of seizure activity (Schecter et al. 1984). Taurine is thought to act as an inhibitory neuromodulator in the CNS, stabilizing the membranes and inhibiting the firing of neurons (Saransaari and Oja 2000). Glycine exerts inhibitory actions, preferentially in the brain stem and medulla (Ure and Perassolo 2000).

Glutamate, for its part, is the most important excitatory neurotransmitter in the human CNS. It is involved in epileptogenesis, initiation and spreading of seizures and seizure- related neuronal damage (Meldrum 1994). Aspartate is also an excitatory amino acid. It may trigger seizures and have a role in maintaining continuous seizure activity, and provokes cell damage (Meldrum 1994; Ure and Perassolo 2000). An increase in the aspartate levels in the brain during seizures has been reported (Carlson et al.1992). Glycine increases the affinity of NMDA receptors for glutamate and in this role it therefore also acts as an excitatory amino acid (Johnson and Asher 1987).

Results from different studies on the amino acid concentrations in human seizure disorders have been markedly variable. In early studies on the cerebrospinal fluid (CSF), the levels of the major excitatory amino acids have been found to be increased (Plum 1974; Engelsen and ElSayed 1984; Kälviäinen et al. 1993), decreased (Mutani et al. 1974; Crawford and Chadwick 1987) and unaltered (Araki et al. 1988; Devinsky et al. 1993). Likewise, the main inhibitory amino acids have been found to be either unaltered (Araki et al. 1988; Pitkänen et al. 1989; Crawford and Chadwick 1993; Devinsky et al. 1993; Kälviäinen et al. 1993) or decreased (Wood et al 1979). Thus, in the light of the previous literature human seizure disorders may not involve any profound changes in the levels of either excitatory or inhibitory amino acids in the CSF.

An elevated glutamate level in the blood plasma has been a consistent finding in previous studies of generalized epilepsy (Van Gelder et al. 1980; Huxtable et al. 1983; Janhua et al. 1992), while the results on localization-related epilepsies have been less consistent. The level of glutamate has been reported to be either increased (Huxtable et al. 1983; Janhua et al. 1992) or unaltered (Monaco et al. 1975; Rao et al. 1993). These inconsistencies may be due partly to heterogeneous patient populations and different AEDs used.

2.1. GABA

2.1.1. Metabolism and receptors

GABA is first synthesized from glutamate by glutamate decarboxylase (GAD) in presynaptic nerve terminals and then released into the synaptic clefts, where it activates postsynaptic receptors. The receptors are either ionotropic $GABA_A$ and

$GABA_C$ receptors, selectively permeable to Cl^-, or metabotropic $GABA_B$ receptors which are G-protein-coupled, diminish the intracellular cAMP levels and open the membrane K^+ channels (Borden 1996). Approximately 15 genes for $GABA_A$ receptor subunits have been identified. The isoforms of $GABA_A$ receptors display variable age- and tissue-specific expression (DeLorey and Olsen 1992; Olsen et al. 1999). Classical pharmacological and ligand binding studies have shown the heterogeneity of $GABA_A$ receptors (Olsen et al. 1999). The action of GABA is terminated by uptake into neurons and glial cells, where GABA transaminase (GABA-T) converts it to glutamate and succinate (Sherif 1994). GABA-T is widely distributed in the CNS, in both neurons and glia cells (Larsson and Shousboe 1990). It is also present in several non-neural tissues such as blood platelets, liver, kidney and heart (White 1979). The activity of GAD is believed to be the major factor in the regulation of the steady-state concentration of GABA in vivo (Bernasconi et al. 1984; Martin 1987). The metabolism of GABA is linked to a substrate cycle (glutamate-GABA-glutamine cycle) between neurons and astrocytes (Schousboe et al. 1992, Shank et al. 1993; Sonnewald et al. 1993) (Fig.1).

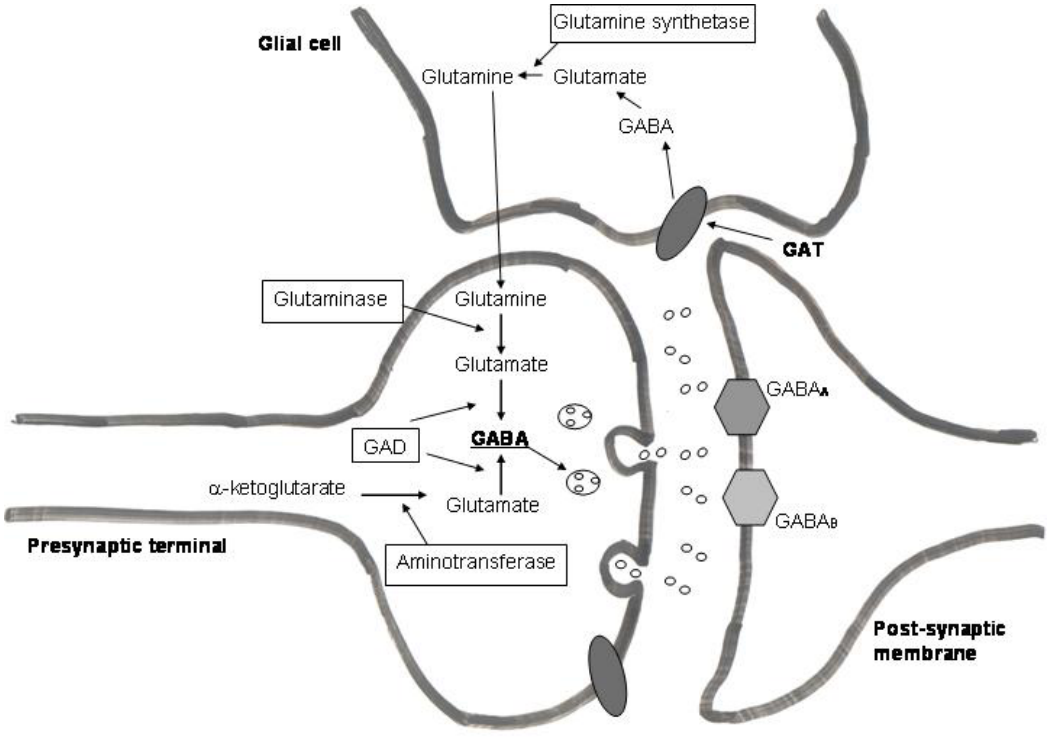

Fig. 1. Events in a GABAergic synapse

α-Ketoglutarate formed in the Krebs cycle is transaminated by GABA transaminase (GABA-T) to glutamate. The transmitter GABA is formed from glutamate by glutamate decarboxylase (GAD). The released GABA is taken up by high-affinity GABA transporters (GAT) present on neurons and glia. GABA receptors are located both pre- and postsynaptically.

2.1.2. GABA transporters

There are four different types of transporters for GABA: GAT-1, GAT-2, GAT-3 and GAT-4/BGT-1. These GABA-transporters may be located heterogeneously in different areas of the human brain and may also manifest themselves differently at different stages during nervous system development (Borden 1996). GAT-2 abounds in the brain of newborn rats, suggesting that it has a role in development (Liu et al. 1993; Borden 1996; Olsen et al 1999). The various subtypes have 50% identity in their amino acid sequence. GAT-1 and GAT-3 are the main subtypes in neurons and glia. Both are restricted to the CNS and thought to be the most relevant to GABAergic neural transmission (Roettger and Amara 1999).

GAT-1 mRNA is discernible in all brain regions, being apparently restricted to the CNS. GAT-2 is present in both the CNS and a number of non-neural tissues. It has also been found in leptomeninges surrounding the brain. It may thus regulate GABA levels in the cerebrospinal fluid (Durkin et al. 1995). GAT-3 is restricted to the CNS (Olsen et al. 1999). In the brain, BGT-1 is widespread but not corresponding to the distribution of GABAergic pathways (Borden et al. 1995). BGT-1 was first identified in many non-neural tissues, kidney, liver, heart and skeletal muscle (Borden et al 1995; Rasola et al. 1995), but later also in the BBB structures in mice (Takanaga et al. 2001). BGT-1 also transports betaine, but its affinity for GABA is about 25-fold higher (Borden et al.1995).

In addition to uptake, GABA transporters can also mediate the release of GABA into the extracellular space, if the intracellular sodium concentration is increased, as during extremely enhanced neural activity (epileptic status) or ischemia (Pin and Bockaert 1989).

None of the GABA transporters has previously been identified in human blood platelets.

2.1.3. Role of GABA in epilepsy

GABAergic neurotransmission has been shown to be changed in both animal and human studies of epilepsy. Brain insults leading to epilepsy harm the GABAergic system. It has also been suggested that GABAergic cells may be most vulnerable to hypoxic insults and epileptic status (Meldrum 1989). GABAergic transmission may be depressed in focal epilepsies (Ribak et al. 1979; Patrylo et al. 2001) and in some models of generalized tonic-clonic epilepsy (Ure et Perassolo, 2000), whereas in generalized absences it may be enhanced (Snead et al. 1990; Ure et Perassolo, 2000). Penicillin is commonly used to cause generalized epileptic seizures. It causes absences, myoclonic seizures and GTCSs (Ostojic et al. 1997; Jimenez et al. 2000). Penicillin is thought to bind to the $GABA_A$ receptor recognition sites and to act as an

antagonist (Ostojic et al. 1997). An important therapeutic approach to enhance the activity of GABA receptors is to inhibit GABA transporters and thus prolong the time during which GABA is present in the synaptic cleft (Roettger and Amara 1999). Several studies have indicated that the clearance of extracellular GABA is compromised in patients with MTLE (During et al. 1995; Williamson et al. 1995). Changes have also been shown in the distribution of GABA transporters in the hippocampus (Mathern et al.1999). GABA transport is functionally impaired in both forward and backward directions in patients with temporal lobe epilepsy and hippocampal sclerosis (TLE+HS) (Patrylo et al. 2001).

The $GABA_A$ receptors are the target of anticonvulsants (benzodiazepines and barbiturates) and also of convulsants (picrotoxin, bicucullin and penicillin) (Ostojic et al. 1997; Olsen et al. 1999). The $GABA_A$ receptors show changes in TLE, both the receptor density and structural properties being altered (Savik et al. 1988; Brooks-Kayed et al.1998; Shumate et al.1998; Olsen et al. 1999; Loup et al. 2000). The binding of ligands to the $GABA_A$ receptors is likewise diminished in patients with TLE+HS, possibly due to cellular loss (McDonald et al. 1991; Olsen et al. 1992). The binding of BZPs to $GABA_A$ receptors is reduced in many other epilepsies but not in CAE or JME (Olsen et al. 1999). In CAE, changes in both $GABA_A$ and $GABA_B$ receptor-mediated neurotransmission have been thought to play a role in the generation of absence seizures (Liu et al. 1991, 1992). On the other hand, the density and affinity of these receptors for GABA is not altered in the brain of genetic absence epilepsy rats from Strasbourg (GAERS) (Knight and Bowery 1992). This epilepsy variant may stem from excessive GABA-mediated inhibition in the thalamus, since there is evidence that basal extracellular GABA is increased in GAERS (Richards et al. 1995). Status epilepticus has been shown to cause down-regulation of the presynaptic $GABA_B$ receptors, which may play a role in the maintanence of seizures and the development of epilepsy by modulating mossy fiber transmission (Chandler et al. 2003).

GABA-T activity in platelets has previously been reported to be decreased in new epileptic patients, especially in those with absences or simple partial seizures (SPS) (Arteaga et al 1993), but to increase in adult patients with epilepsy (Armijo et al.1989; Kumlien et al. 1995). However, Sherwin (1999) found the activity of GABA-T in the human epileptogenic cortex to be unaltered.

2.2. Glutamate

2.2.1. Glutamate metabolism and receptors

Glutamate is a non-essential amino acid produced from 2-oxoglutarate, glutamine, ornithine or proline. At least the greater part of this neurotransmitter is formed from glutamine by glutaminase. It is released into the synaptic cleft, where it activates postsynaptic receptors and is mainly taken up by transporters to glial cells (Fig. 2). The intracellular level of glutamate is lower in glial cells than in neurons due to the rapid conversion of glutamate to glutamine by glutamine synthetase in the former (Rothstein et al. 1996). The glutamate receptors can be divided into two categories. (1) ionotropic N-methyl-D-aspartate (NMDA), 2-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) and kainate receptors, which act through Na^+ or Na^+ and Ca^+ channels and (2) metabotropic receptors, which are associated with G-proteins and either activate phospholipase C or decrease the activity of adenylyl cyclase (Tanabe et al. 1992). The NMDA receptors are further divided into NR1, NR2A, NR2B, NR2C, NR2D receptors, the AMPA receptors into GluR1-4 and the kainate receptors into GluR5-9, KA1 and KA2. The metabotropic mGluR1-8 receptors can be divided into 3 different groups (Meldrum 1994; Meldrum and Chapman 1999; Chapman 2000; Danbolt 2001).

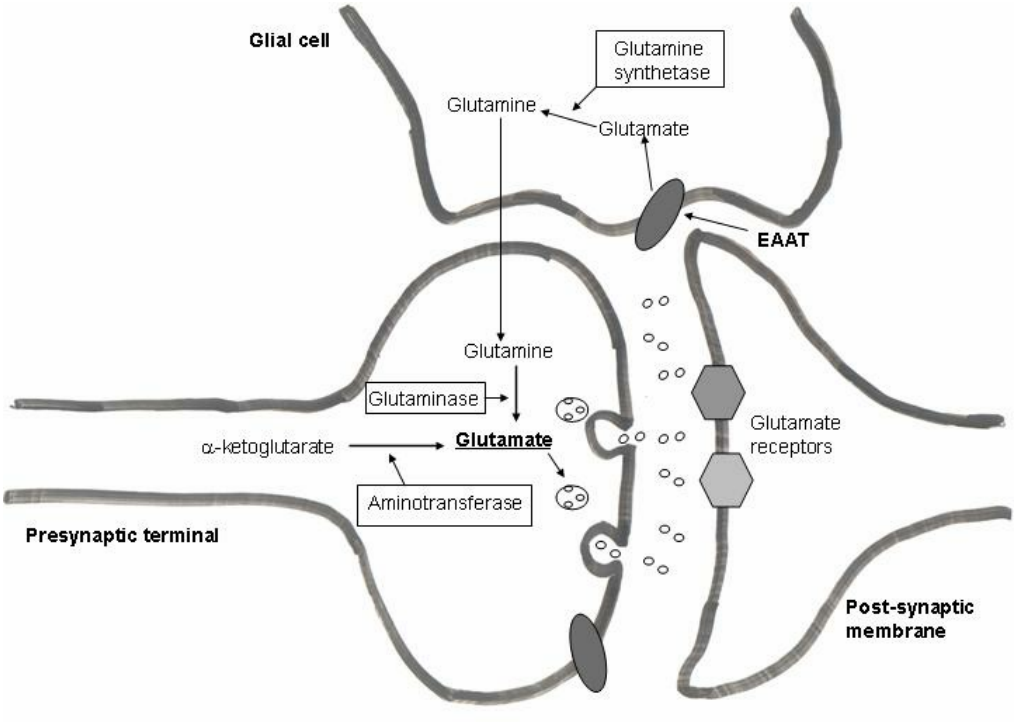

Fig. 2. Events in the glutaminergic synapse

Glutamate, synthesized via metabolic pathways, is concentrated by a vesicular transporter into secretory granules. After release from the presynaptic terminal, glutamate can interact with its receptors. It is then cleared from the synaptic cleft by transporters or by recycling through the adjacent glia.

2.2.2. Glutamate transporters

Since glutamate has no extracellular metabolizing enzyme, uptake into neurons and glial cells is the mechanism to terminate its action (Chapman 2000; Danbolt 2001; Gegelashvili et al. 2001). Five different excitatory amino acid transporters (EAATs), named EAAT1-5, have been cloned in humans (Danbolt 2000). These transporters act through Na^+ and K^+ channels. The EAAT subtypes differ in their regional, cellular and developmental distribution. Different subtypes are 50-60% identical with respect to their amino acid sequence.

EAAT1 (GLAST) is present mainly in cerebellar glia but also in many peripheral organs and tissues (Danbolt 2001; Arriza et al. 1994; Fairman et al. 1995). It is the dominant glutamate transporter in the cerebellum and circumventricular organs. Astroglial cells express GLAST protein and GLAST mRNA. There is no evidence of neuronal GLAST.

EAAT2 (GLT-1) is the major glutamate transporter and abounds in astrocytes in the hippocampus and cerebral cortex. Glial cells express GLT protein while neurons have so far been shown to express only GLT mRNA. EAAT2 has also been demonstrated to be present in the placenta (Arriza et al. 1994; Fairman et al. 1995).

EAAT3 (EAAC1) is found in the cerebral cortex, hippocampus and caudate-putamen, but its role in the regulation of uptake is only minor. EAAT3 is highly neuron-specific in the CNS and it is thought to be postsynaptic (Rothstein et al. 1994). It is also detectable in peripheral tissues. The share of EAAT3 in glial glutamate transport has been estimated to be 20-40% (Rothstein et al. 1996). EAAT4 is only present in cerebellar Purkinje cells and EAAT5 only in the retina (Proper et al. 2002).

GLT and GLAST are responsible for most of the glutamate uptake in the human CNS. Even though both neurons and glia have glutamate transporters, it is commonly thought that the uptake capacity of astrocytes is higher than that of neurons, and astrocytes have therefore the major role in glutamate removal (Rothstein et al. 1996).

Human platelets have been shown to express EAAT1, EAAT2 and EAAT3 transporters but all at lower densities than in the brain. All transporters have been found in both platelet membranes and cytoplasm (Zoia et al. 2004).

2.2.4. Role of glutamate in epilepsy

Glutamate is involved in epileptogenesis, the initiation and spreading of seizures, and seizure-related neuronal damage. The NMDA receptors are believed to have

the major role in these phenomena (Obrenovitch and Urenjak 1997). The density of NMDA, AMPA and kainate receptors has been shown to be increased in epileptogenic tissue (Geddes et al. 1990; Hosford et al. 1991; McDonald et al. 1991). The properties of NMDA and AMPA receptors have been found to change in human epilepsy (Musshoff et al. 2000). Humans with MTLE have an increased number of NMDA receptors in the molecular layer and mossy fiber sprouting is associated with physiologically active NMDA receptors (Mathern et al. 1997). Furthermore, the NMDA and AMPA receptor antagonists are powerful antiepileptic drugs in animal models (Meldrum and Chapman 1999). On the other hand, glutamate receptor agonists are convulsants and injections of AMPA, NMDA and kainate induce seizures (Obrenovitch and Urenjak 1997). The kainite-induced seizures cause selective loss of hippocampal neurons, providing a model for the sclerosis encountered in temporal lobe epilepsy (Gruenthal et al.1986). The mRNA and protein levels of EAAT3 transporter have been shown to be increased in hippocampal and neocortical neurons in human epilepsy (Crino et al. 2002; Proper et al. 2002), whereas the expressions of EAAT1 and EAAT2 show either no change in epileptic patients (Tessler et al. 1999; Crino et al. 2002) or only in some specific regions of the hippocampus in temporal lobe epilepsy (Mathern et al. 1999; Proper et al. 2002). In trauma-induced epilepsy the levels of mRNA of EAAT1, EAAT2 and EAAT3 have been increased during the acute phase, whereas during the chronic periods the EAA1 and EAAT2 levels fall and the EAAT3 level increases (Doi et al. 2000; Samuelsson et al. 2000, 2003). There is evidence that a decreased expression of glutamate transporters can lead to seizures (EAAC knockdown, GLT knockout) (Meldrum and Chapman 1999).

Glutamate levels increase in patients with epilepsy in both plasma and the brain (Janhua et al. 1992; During and Spencer 1993; Rowley et al.1995; Ferrie et al 1999).

3. Platelet model

Blood platelets are anucleate disc-shaped cells which originate from megakaryocytes in the bone marrow. The megakaryocyte cytoplasm divides into platelets and platelets are subsequently released into the blood circulation, where they remain up to 9-10 days (Rodgers 1999; Camacho and Dimsdale 2000). Their main function is in hemostasis. Platelet cytoplasm also contains several important organelles. Their dense bodies have high concentrations of serotonin, adenosine diphosphate, adenosine triphosphate and calcium. The platelets also express receptors for benzodiazepines and serotonin (Camacho and Dimsdale 2000) and platelets were first used as a model to study amine transmitters such as serotonin. The uptake of serotonin in platelets models the uptake in neurons (Paasonen 1968; 1973; Lingjaerde 1969). Platelets also have uptake mechanisms

for several amino acid transmitters, among them GABA, glutamate, aspartate and glycine. Platelets have long been used in studies of many neurological and psychiatric diseases (Zieve and Solomon 1968; Airaksinen 1979; Mangano and Schwarcz 1981b; Hambley and Johnston 1985; Ferrarese et al. 1999, 2000, 2001a).

The uptake of glutamate in platelets resembles that in the human CNS (Mangano and Schwarcz 1981a; Ferrarese et al. 1999, 2000). The platelets have recently been shown to express three transporters for glutamate which also exist in the human brain (Zoia et al. 2004). GABA uptake is Na^+- and temperature-dependent in platelets (Airaksinen 1979; Hambley and Johston 1985). The kinetic parameters in platelet GABA uptake are similar to those in the uptake in the CNS and the uptake properties appear to resemble the properties of glial uptake (Hambley and Johnston 1985).

GABA-T is found in both neurons and glial cells in the CNS (Larsson and Shousboe 1990). In addition to the brain, GABA-T is present in many peripheral tissues such as kidney, liver and blood platelets (White and Sato 1978; Sherif and Ahmed 1995). There is no GABA-T in platelet-devoid blood plasma nor in other blood elements (White 1979). The enzyme activity in the platelets is lower than in the brain, but the other properties of platelet GABA-T resemble brain GABA-T (White 1979; Arteaga et al. 1993; Sherif 1994).

Earlier studies on platelets have shown glutamate uptake to be decreased in patients with amyotrophic lateral sclerosis, Down syndrome and Alzheimer's and Parkinson's diseases (Ferrarese et al. 1999, 2000, 2001a; Begni et al. 2003; Zoia et al. 2004). GABA uptake in platelets is likewise reduced in patients with Down syndrome (Enns and McCoy 1980). In an earlier study of patients with progressive myoclonic epilepsy no change was seen in GABA uptake (Airaksinen 1979), but the platelet GABA-T activity was altered in epilepsy (Arteaga et al. 1993; Kumlien et al. 1995).

Prior to this present series the platelet model has been used sparsely in studies of human epilepsy, although it could be an easy and reliable means of characterizing neurotransmitter functions.

AIMS OF THE STUDY

The aims of the study were the following:

1. to measure GABA-T activity and GABA uptake in different epilepsy syndromes using the platelet model,

2. to measure glutamate uptake in different epilepsy syndromes using the platelet model,

3. to establish whether possible changes in the amino acid concentrations in blood plasma or CSF are related to acute seizures and/or epilepsy syndromes, and

4. to establish which GABA and glutamate transporters operate in blood platelets.

MATERIAL AND METHODS

1. Patients

1.1. Adult patients

Adult patients with JME (I, III, IV), RLE (I, IV) or temporal lobe epilepsy with hippocampal sclerosis (TLE+HS) (III, IV) and their age- and sex-matched controls constituted the study cohort. The criteria of ILAE (1989) were used to diagnose JME and TLE, respectively. The diagnostic criteria for RLE patients were refractory seizures despite at least two AEDs at appropriate doses. We also studied patients with recent (within 24 hours) GTCS (IV), which did not necessarily have any epilepsy diagnosis. The patients were recruited from the outpatient Department of Neurology, Tampere University Hospital.

1.2. Children

The patients with typical CAE (II) were recruited from the Children's Neurological Clinic of Tampere University Hospital. Neurologically healthy children treated in the hospital for other reasons served as their controls. The diagnostic criteria of ILAE (1989) were used to diagnose CAE.

1.3. Ethical aspects

The Ethics Committee of Tampere University Hospital approved all studies on patients and volunteers (I-IV). All patients (I, III, IV) or their parents (II) and volunteers gave written informed consent.

2. Samples

Venous blood samples were collected into tubes containing ethylenediaminetetra-acetic acid (EDTA) as anticoagulant. The samples were processed as described by Kumlien and associates (1995) (I, II) or by Mangano and Schwarcz (1981b) (III) with slight modifications. The tubes, cooled in ice, were centrifuged at 200 g at 4 ºC for 10 min. The platelet-rich plasma was then

collected and centrifuged at 2500 or 7000 g at 4 ºC for 10-15 min and the pellets suspended in 0.7 ml buffer (0.1 M sodium phosphate, 0.1 mM EDTA, 0.5 mM dithiothreitol (DTT) and 0.1 mM pyridoxal phosphate; pH 8.4) or in 2.0 ml of ice-cold 0.32 M sucrose. After centrifugation at 7000-10 000 g at 4 ºC for 5-15 min, the platelets were resuspended in 0.35 ml of the same buffer or in 0.32 M sucrose to one fifth of the original plasma volume. The samples were then briefly sonicated on ice (I, II).

The amino acid concentrations in plasma (IV) were measured from blood samples taken into heparin-containing tubes. The cells were centrifuged down at 200 g at 4 °C for 10 min and plasma collected. The plasma samples were stored at –20° C until analyzed.

The lumbar CSF samples (IV) were tapped between 9 a.m. and 2 p.m. The first 2 ml of CSF was used for routine clinical tests and the subsequent 200 µl for the study. The blood samples from the same patients were drawn within 10 min of the lumbar puncture into Vacutainer EDTA vacuum tubes. The CSF samples were stored at – 70 °C until analyzed.

3. Assays

3.1. GABA transaminase activity

GABA-T activity was measured using [^{14}C]GABA as substrate (White 1979; Kumlien et al. 1995). Briefly, 0.68 mM 2-oxoglutarate, 0.1 mM EDTA, 0.5 mM DTT, 0.1 mM pyridoxal phosphate, 18 µM [^{14}C]GABA (NEN Research Products, Boston MA, specific activity 8.3 TBq/mol) and 0.1 M sodium phosphate buffer (pH 8.4) were admixed to the samples The mixtures were incubated at 37 ºC for 30 min. The reaction has been shown to be linear for at least 2 hours (Arteaga et al. 1993). It was terminated by adding ice-cold 1 M HCl, whereafter the mixtures were passed through 0.8 x 4 cm ion-exchange columns and the appropriate eluent fractions counted for radioactivity. All results were corrected by subtracting the radioactivity obtained in blank samples in which 2-oxoglutarate was replaced by the buffer used in the preparation of platelets.

3.2. GABA uptake

GABA uptake by the platelet preparation was estimated with [^{3}H]GABA (Amersham, Bristol, U.K., specific activity 3.26 PBq/mol) at concentrations of 5-500 µM by the method of Hambley and Johnston (1985). The samples were first pre-incubated with modified Krebs-Henseleit buffer (lacking Mg^{2+} and

Ca^{2+}), pH 7.4 for 15 min under 5% CO_2/95% O_2 in a shaking water bath at 37 °C. The uptake was initiated by adding 200 nM [^{3}H]GABA and stopped after 10 min by adding 500 µl of cold saline followed by immediate centrifugation at 10 000 g for 10 min. The pellets were washed and extracted with H_2O and the radioactivity counted. The measurements were made within 6 h from sampling. The results were corrected by subtracting the radioactivity in the unincubated samples. The uptake was linear within the protein content of 50-500 µg for at least 20 min.

3.3. Glutamate uptake

Glutamate uptake by the platelet preparations was measured with L-[^{3}H]glutamate (Amersham, Bristol, U.K., specific activity 1.55 PBq/mol) at concentrations from 5 to 500 µM (Mangano and Schwarcz 1981b). Briefly, the samples were first preincubated with Tris-citrate buffer, pH 7.4, for 15 min under oxygen in a shaking water bath at 37 °C. Then, 0.7 µM [^{3}H]glutamate was added and the incubation stopped after 10 min by adding 500 µl of cold saline followed by immediate centrifugation for 10 min at 10 000 g. The pellets were washed twice, extracted with H_2O and counted for radioactivity. The assays were done within 6 h after taking blood samples. The results were corrected by subtracting the radioactivity in the unincubated blank samples. The uptake was linear within the protein content of 50-500 µg for at least 20 min.

In order to evaluate the breakdown of radioactively labeled glutamate during the uptake experiments, a number of samples were subjected to thin-layer chromatography using n-butanol-acetic acid-water (80:20:20) as solvent. The breakdown of L-[^{3}H]glutamate was found to be negligible during the experiments, since no significant amount of radioactivity was detected outside the glutamate spot.

3.4. Amino acid concentrations

The amino acid concentrations in plasma were measured from blood samples taken into heparin-containing tubes. The cells were centrifuged down at 200 g at +4 °C for 10 min and plasma was collected. The plasma samples were stored at -20° C until analyzed. Thereafter, 500 µl samples were deproteinized with 50 µl of 50% sulfosalicylic acid containing 50 µl of 5 mM DL-diamino-n-butyrate as internal standard. These tubes were left to stand for an hour at +4°, whereafter they were centrifuged for 10 min at 16 000 g. An aliquot of 300 µl of the supernatant was admixed with 175 µl of 0.2 M lithium citrate buffer (pH 2.2) and 25 µl of saturated LiOH. The mixtures were subjected to ion-exchange chromatography using an automatic Shimadzu LC-10AD amino acid analyzer

with o-phthalaldehyde derivatization and a Shimadzu fluorescence RF-AXL detector.

3.5. In vitro analyses with antiepileptic drugs

To study the effects of VPA, CBZ, LTG and ESM on GABA and glutamate uptake or on GABA-T activity in platelets from healthy volunteers, 150-1200 μM VPA, 10-80 μM CBZ, 1-100 μM LTG and 20-160 μM ESM were added to the incubation mixtures 5 min prior to the addition of platelets. The assays were done in triplicate.

3.6. Isolation of mRNA from human platelets

Enriched platelets obtained from healthy adult human volunteers were used. The total RNA was first isolated with Trizol reagent (Gibgo BRC, Gaithesburg, MD). Ten milliliters of Trizol were used to isolate RNA from about 8.4 x 10^{10} cells. Three to five hundred micrograms of total RNA obtained in this way were then further purified with poly d(T) cellulose. The RNA sample was first diluted to 10 ml of tissue lysis buffer containing 200 mM NaCl, 200 mM Tris (pH 7.5), 1.5 mM $MgCl_2$, 2% sodium dodecyl sulfate (SDS) and 200 mg/l proteinase K (Fermentas, Vilnius, Lithuania) in diethylpyrocarbonate (DEPC) -treated water. The solution was passed 4 times through a sterile 21-gauge needle and thereafter incubated in a water bath at 45 °C for 60 min. NaCl was added to each sample to a final concentration of 500 mM. The samples were then passed 4 times through a 21-gauge needle. Oligo d(T) cellulose (50-75 mg; Sigma Genosys Ltd, Haverhill, UK) was added to each sample and the samples were incubated at room temperature for 60 min. They were then centrifuged at 10 000 g for 10 min at 4 °C and the supernatant removed. The pellets were washed twice in a buffer containing 500 mM NaCl and 10 mM Tris (pH 7.5) in DEPC-treated water and then three times in a buffer containing 250 mM NaCl and 10 mM Tris (pH 7.5) in DEPC-treated water. The samples were transferred into spin-columns (Vivaspin concentrator; Vivascience AG, Hannover, Germany) and washed four times with a buffer containing 250 mM NaCl and 10 mM Tris (pH 7.5) in DEPC-treated water. Messenger RNA was eluted with 400 μl of buffer containing 10 mM Tris (pH 7.5) in DEPC-treated water. The eluate was then incubated at 37 °C for 30 minutes with 60 IU of RNAse inhibitor (Fermentas, Vilnius, Lithuania) and 5 IUs of RNAse-free DNAse (Boehringer Mannheim Gmbh, Mannheim, Germany). The mRNA was precipitated with ethanol, dissolved in water and stored at -80 °C until used. The concentration of mRNA was determined with Picogreen reagent (Molecular Probes, Eugene, MO, USA). The yield of this isolation was about 8 μg of mRNA.

3.7. Reverse transcription-polymerase chain reaction

The smart RACE cDNA amplification kit (Clontech, Palo Alto, CA, USA) was used for reverse transcription-polymerase chain reaction (RT-PCR). One microgram of mRNA was reverse-transcribed with poly(dT) primer and Powerscript reverse transcriptase. The reaction was made in 1.5 h at 42 °C in a volume of 20 µl, after which the RT-PCR library obtained was diluted with 50 µl of dilution buffer containing 10 mM Tricine-KOH and 0.1 mM EDTA. Five microliters of this library was used for each PCR reaction. The PCR reaction was first denatured at 94 °C for 30 s. The annealing time was 30 s at 62 or 64 °C and elongation 1 or 1.5 min at 72 °C. The fragments were analyzed in 1.2% agarose gel. The bands produced were eluted from the gel with a Qiaex II gel extraction kit (Qiagen, Chatsworth, CA, USA) and cloned into pCRII vector (Invitrogen, San Diego, CA, USA). Plasmid DNA was extracted using the Qiaprep spin miniprep kit (Qiagen, Chatsworth, CA, USA) kit. The sequencing reaction was performed with the BigDye terminator version 3.1 cycle sequencing kit and the sequencing analysis with an Abi 310 gene analyzer.

3.8. Protein measurements

Protein was measured by the method described by Lowry and colleagues (1951).

4. Calculation of kinetic parameters

The uptake velocities of GABA and glutamate in the concentration range of 5-500 µM were fitted (Program Fig.P for Windows, version 2.2a) with the equation $v = V_m \times s/(K_m+s)$, in which v is the velocity of uptake, V_m the maximal velocity, K_m the Michaelis constant and s the GABA or glutamate concentration in medium.

5. Statistical analysis

Comparisons of different patients groups were made by Student's unpaired t-test in the case of normal distribution and Mann-Whitley U-test when analyzing unevenly distributed data. The results are presented as mean ± standard deviation (S.D.) or standard error mean (S.E.M.).

RESULTS

1.1. GABA transaminase activity in blood platelets from adult patients with juvenile myoclonic epilepsy or refractory partial epilepsy (I) and in children with typical childhood absence epilepsy (II)

The GABA-T activity in platelets was elevated in the adult patients with JME, while patients with RLE did not differ from the controls (Table 2). The GABA-T activity was lowered in children with CAE, regardless of the medication used (Table 3).

Table 2. GABA-T activity (μmol/min/kg protein) in adult epileptic patients

	GABA-T activity	
Controls	0.67 ± 0.30	
JME	0.94 ± 0.39	p< 0.001
RLE	0.69 ± 0.29	

Significance compared to controls. Mean values ± S.D.

Table 3. GABA-T activity (μmol/min/kg protein) in children with CAE

	GABA-T activity	
Controls	1.75 ± 0.10	
CAE patients	1.22 ± .0.05	P<0.001
ESM	1.08 ± 0.06	P<0.001
VPA	1.35 ± 0.05	P<0.01

Significance compared to healthy controls. Mean values ± S.D.

1.2. GABA uptake in adult patients with juvenile myoclonic epilepsy, refractory partial epilepsy (I) and in children with typical childhood absence epilepsy (II)

The uptake was optimal in pH 7.4. It was sodium-dependent and saturable. Kinetic analyses revealed only one component in each epilepsy syndrome studied. The maximal capacity of GABA uptake into platelets was decreased in both adult patient groups JME and RLE, the change being greater in patients with JME (Table 4). In children with CAE the maximal capacity of GABA uptake into platelets was greater in the VPA group but not in the ESM group (Table 5). The

affinity of the transporters for GABA was not significantly altered in any patient groups, JME, RLE or CAE.

Table 4. Kinetic parameters of GABA uptake in patients with JME or RLE

	Vm (µmol/min/kg protein)		Km (µM)
Controls	2.27 ± 0.15		613 ± 64
JME	1.48 ± 0.09	P<0.001	471 ± 100
RLE	1.84 ± 0.07	P<0.05	569 ± 68

Significance compared to controls. Mean values ± S.E.M.

Table 5. Kinetic parameters of GABA uptake in children with CAE

	Vm (µmol/min/kg protein)		Km (µM)
Controls	0.26 ± 0.06		97.2 ± 28.7
All patients	0.53 ± 0.05	P<0.01	93.0 ± 12.6
ESM	0.34 ± 0.05		80.3 ± 19.9
VPA	0.66 ± 0.09	P<0.01	91.3 ± 19.8

Significance compared to controls. Mean values ± S.E.M.

1.3. Glutamate uptake (III) in patients with juvenile myoclonic epilepsy or temporal lobe epilepsy with hippocampal sclerosis

The uptake was found to be optimal in pH 7.4 and sodium-dependent. It was saturable. Kinetic analyses revealed only one component in both epilepsy syndromes studied. Glutamate uptake was enhanced at low glutamate concentrations in patients with TLE+HS when compared to controls. The maximal velocity of transport was not significantly altered. In patients with JME, the transport parameters did not differ from those of the volunteers (Table 6).

Table 6. Kinetic parameters of glutamate uptake in patients with JME or TLE+HS

	Vm (µmol/min/kg protein)	Km (µM)	
Controls	0.50 ± 0.07	118.7 ± 17.2	
JME	0.35 ± 0.04	80.3 ± 13.1	
TLE+HS	0.36 ± 0.06	49.5 ± 9.7	P<0.01

Significance compared to controls. Mean values ± S.E.M.

1.4. Amino acid concentrations in blood plasma (IV)

Patients with acute seizures had significantly lower plasma levels of most amino acids when compared to the control subjects, whereas the levels of glutamate and aspartate were significantly increased. In JME patients, the plasma levels of glutamate, glycine, serine and alanine were significantly elevated and those of glutamine, asparagine, phenylalanine, histidine and tryptophan reduced when compared to the controls. RLE patients had significantly lower plasma levels of a number of amino acids but a higher level of tyrosine when compared to the controls. The plasma level of citrulline was lowered in all patient groups when compared with the controls. The plasma concentrations of aspartate, glutamate, glycine, serine, alanine, isoleucine, leucine and lysine were significantly lower in RLE than in JME patients, and the levels of glutamine, cystine, tyrosine, histidine and tryptophan were significantly higher in RLE than in JME patients . GABA was not detectable in plasma (IV, Table II).

1.5. Amino acid concentrations in cerebrospinal fluid (IV)

The CSF amino acid concentrations were measured after acute GTCSs. In the CSF, the levels of taurine and ornitine were significantly lower and that of phenylalanine significantly higher in the patients than in control subjects (IV, Table III).

1.6. In vitro experiments with antiepileptic drugs

The in vitro analyses were made at clinically relevant concentrations of AEDs most commonly used in our patient population. ESM showed that GABA uptake by the platelets diminished in a concentration-dependent manner at clinically relevant ESM concentrations, although this change does not appear to have affected the results. None of the other drugs tested induced any marked changes in either GABA-T activity or in GABA and glutamate uptake (Table 7).

Table 7. Absence of in vitro effects of antiepileptic drugs

AED (concentrations)	Effect on GABA uptake	Effect on glutamate uptake	Effect on GABA-T activity
CBZ (10-80 μM)	-	-	-
ESM (20-160 μM)	↓	n.t.	-
LTG (1-100 μM)	-	-	-
VPA (150-1200 μM)	-	-	-

- = no effect; n.t. = not tested; ↓ = decreased.

1.7. GABA and glutamate transporters in platelets (V)

Since blood platelets do not contain nuclei, a large amount of platelets was needed to obtain enough mRNA. For RT-PCR we used primers designed to recognize all transporters for GABA and glutamate. The BGT-1 transporter for GABA and the EAAT3 transporter for glutamate were cloned from human platelets.

DISCUSSION

1. Changes in GABA metabolism in epileptic patients

GABA-T activity was found to be changed in two different types of IGEs but not in the localization-related epilepsies. Patients with CAE had low GABA-T activities but those with JME elevated GABA-T activities. In general, our results are in agreement with those reported in previous studies. Arteaga and colleagues (1993) have found GABA-T activity to be increased in myoclonic epilepsies and decreased in untreated absence epilepsies. The findings in localization-related epilepsies have hitherto been inconsistent. There is one study in which no difference was found between patients and controls (Arteaga et al. 1993) and one study in which the platelet GABA-T activity in patients with RLE was increased when compared to the controls (Kumlien et al. 1995).

Our observations suggest that changes in the activity of platelet GABA-T may be a marker for some generalized epileptic syndromes. The findings also support the hypothesis that genetic factors may play a major role in determining the activity of platelet GABA-T (Berrettini et al. 1982), because changes were only seen in the idiopathic epileptic syndromes and the change was different in these two different IGE types.

The level of GABA-T activity was in general higher in children than in adults. In previous studies on humans (Armijo et al. 1989; Sherif et al. 1992) there have been no age-related differences. On the other hand, in animal studies GABA-T activity has been seen to increase two-fold during the first weeks of life, decreasing thereafter (Sherif et al. 1993; Sherif 1994).

The capacity of GABA uptake was decreased in adult JME and RLE patients, although the difference was more pronounced in the JME patients. Children with CAE exhibited an increase in the maximal capacity of GABA uptake, but only in those patients treated with VPA. The affinity of GABA uptake was not altered in any patient group.

The capacity of GABA uptake was markedly higher and the affinity significantly lower in adults than in children. Animal studies have shown that the low-affinity GABA uptake increases and the high-affinity uptake decreases in the brain with age (Oja and Kontro 1983). The present age-related difference in human platelets seems thus to be logical. BGT-1 was the only GABA transporter found to be functioning in blood platelets. There is evidence from animal studies that BGT-1 is a low- affinity transporter of GABA while the other known GABA transporters are

high-affinity transporters (Borden 1996; Gadea 2001). Our kinetic analyses are in keeping with these animal data since the present estimated kinetic properties of GABA uptake indicate low-affinity transport. BGT-1 has also selectivity for betaine (Borden 1996). BGT-1 has been found in the mouse blood-brain barrier (BBB), suggesting that it is involved in GABA transport across the BBB and may compensate for the reuptake of GABA in neuronal and glial cells (Takanaga et al. 2001). A part of transport may also represent the contribution of taurine transporters. The taurine transporter was now discernible in human platelets (V) and this carrier also transports GABA (Sivakami et al. 1992).

2. Changes in glutamate metabolism in epileptic patients

There is an abundance of experimental and clinical data to support the conception that seizures are associated with elevated brain glutamate levels (Janhua et al. 1992; Wilson et al. 1996; During and Spencer 1993). The NMDA receptors are traditionally thought to have a major role in epileptic changes (Obrenovitch and Urenjak 1997). They are readily activated in the epileptic kindling model (Mody et al. 1988). An increased binding of ligands to NMDA receptors and an increase in the density of NMDA receptors have been demonstrated in TLE (McDonald et al. 1991; Roper et al.1992). Glutamate uptake has been shown to increase in animal models of epilepsy (Ortiz et al. 1996; Lewis et al. 1997). Glutamate uptake was now enhanced in the patients with TLE+HS when compared to the controls. The patients with IGE did not differ from the controls. To our knowledge there are no previous studies on glutamate uptake in human epilepsies.

The up-regulation of glutamate uptake in the patients with TLE+HS could be a compensatory mechanism to control high glutamate levels resulting from seizure activity. Some previous studies have shown that glutamate levels increase due to seizures (Janhua et al. 1992, Wilson et al. 1996) and we likewise noted that the glutamate levels increase in plasma after acute seizures. On the other hand, the glutamate levels in our patients with localization-related epilepsy did not increase during the interictal phase, which is in keeping with our assumption that the enhanced uptake is a compensatory mechanism. This altered uptake does not appear to be sufficient to prevent excitotoxicity due to the high glutamate levels incurred.

The EAAT3 transporter of glutamate was cloned from platelets. Quite recently, Zoia and colleagues (2004) cloned three EAAT transporters from blood platelets. We could not confirm their discoveries on EAAT 1 and EAAT2. However, our results would indicate that platelets provide an applicable model for glutamate uptake in the CNS.

3. Amino acid changes in epileptic patients

Although we have no control values for acutely seizured patients obtained from themselves, the levels of most amino acids in plasma seem to be lowered after acute tonic-clonic seizures, while those of the excitatory amino acids glutamate and aspartate are increased when compared to controls. These observations indicate changes in the metabolism of these amino acids in human seizure disorders, leading to an imbalance between excitatory and inhibitory amino acids. To the best of our knowledge, this study is the first on the plasma levels of amino acids after acute tonic-clonic seizures. Interestingly, the concentration of glutamate has been reported to be increased in the CSF and blood plasma in two other syndromes, stroke and human immunosuppressive virus dementia, being presumably related to excitotoxic neuronal damage (Castillo et al. 1996; Ferrarese et al. 2001).

In the CSF, no changes were seen in the levels of excitatory amino acids. The results from previous studies on the excitatory amino acids in the CSF of epileptic patients are markedly inconsistent. The interictal levels of glutamate and aspartate have been reported to be increased, reduced (Mutani et al. 1974; Plum 1974; Engelsen and ElSayed 1984; Crawford and Chadwick 1987; Kälviäinen et al. 1993) or unchanged (Araki et al. 1988; Devinsky et al.1993). Of previous human studies, the most relevant from the standpoint of our present investigation are those of Pitkänen and colleagues (1989) and Devinsky and colleagues (1993), in which no differences were found in the interictal and postictal levels of glutamate or aspartate. Our results thus corroborate the assumption that human seizures are not associated with any significant change in the CSF markers of excitatory amino acid neurotransmission.

The level of the inhibitory amino acid taurine was lowered in the CSF after seizures, a result in keeping with that of Crawford and Chadwick (1987), but not with some other studies (Mutani et al. 1974; Araki et al. 1988; Devinsky et al. 1993; Kälviäinen et al. 1993; Pitkänen et al. 1989). The level of the most important inhibitory amino acid GABA was not altered in the CSF after acute seizures. This finding is in agreement with previous studies on newly diagnosed untreated patients and on chronic patients examined during the interictal and postictal states (Crawford and Chadwick 1987; Pitkänen et al. 1987; Araki et al. 1988; Pitkänen et al. 1989; Devinsky et al. 1993; Kälviäinen et al. 1993). Wood and co-workers (1979) have reported that the GABA levels decrease in patients with intractable epilepsy. We conclude that human seizure disorders would not appear to be associated with any major alterations in the inhibitory amino acids in the CSF.

In patients with JME the main finding was an increase in the plasma glutamate level. This observation is in agreement with those in previous studies on other generalized epileptic syndromes (Van Gelder et al. 1980; Huxtable et al. 1983;

Janhua et al. 1992). This increase might result from alterations in glutamate transport, uptake or metabolism. No such alteration in glutamate uptake was seen in patients with JME (III). VPA has been shown to increase the levels of glycine, alanine and serine in blood serum (Iinuma et al. 1988). Most of our JME patients used VPA. We saw an elevation in glycine and serine but not in alanine in our patients.

In patients with localization-related epilepsies the levels of excitatory amino acid aspartate and glutamate were reduced, but no changes were discernible in the inhibitory amino acids when compared to the patients with JME. Previous studies of localization-related seizures have been somewhat variable, partly due to differences in patient populations and AEDs used. The plasma level of glutamate has been reported to be increased or unaltered (Monaco et al. 1975; Huxtable et al. 1983; Janhua et al. 1992; Rao et al. 1993).

Our results suggest that alterations in the amino acid levels in plasma are related to epileptic syndromes and time-related to the seizures themselves.

4. Effects of antiepileptic medications

Several AEDs affect GABA and glutamate functions either directly or indirectly. We excluded from the GABA studies all patients who used drugs known to have a direct effect on GABA metabolism. These drugs were vigabatrin, a GABA-T inhibitor, and tiagabine, an inhibitor of GABA uptake. The other AEDs most commonly used in our patient material were CBZ, ESM, LTG and VPA. Their effects on the results obtained were also taken into account.

CBZ is a conventional antiepileptic which works through Na^+ channels. It is effective in partial and generalized seizures but may aggravate myoclonic and absence seizures. CBZ has previously been shown in human and animal studies, both in vivo and in vitro, not to affect GABA-T activity (Battistin et al. 1984; Kumlien et al. 1995). CBZ was also now shown not to alter GABA and glutamate uptake or GABA-T activity.

ESM has a specific mechanism of action through the T-type Ca^{2+} channels (Keränen et al. 1997). It is used only in absence seizures. Here it did not alter GABA-T activity. Only a slight inhibition of GABA-T at high concentrations has previously been reported (Löscher, 1980). ESM at high concentrations was here found to diminish GABA uptake in platelets. To our knowledge no previous studies have been undertaken on the ESM effect on GABA uptake.

LTG is a newer antiepileptic which exerts an effect on Na^+ and Ca^{2+} channels. It is thought to forestall the release of excitatory neurotransmitters (Morrell 2002; White 2003). It can be used in both partial and generalized epilepsies. This drug

likewise did not affect GABA and glutamate uptake, nor was any change found in GABA-T activity. Chronic LTG treatment has previously been shown to increase the level of hippocampal GABA, this leading to a secondary increase in the GABA-T activity (Hassel et al. 2001).

Although VPA is regarded as an old AED, its mechanism of action is still not completely known. It is apparently a drug of multiple sites of action. It affects both Na^+ and Ca^{2+} channels and elevates the brain GABA levels. The midbrain regions which play a critical role in seizure generation and propagation show the most marked changes in GABA after VPA treatment (Löscher 1989). The drug is effective in all types of seizures and epilepsy syndromes. No effect on GABA and glutamate uptake and only a trend towards decreased GABA-T activity at high concentrations of VPA were now seen in platelets. In accord with our findings, Kumlien and coworkers (1995) have proved that VPA does not alter in vitro the GABA-T activity, and Larson and associates (1986) have noted an inhibition of GABA-T in astrocytes at high concentrations of VPA. In animal studies VPA has been shown not to affect glutamate uptake (Nilsson et al. 1992) but to have an effect on the affinity but not on the transport capacity of GABA uptake (Nilsson et al 1990; 1992). There are also several studies indicating that VPA does not affect GABA uptake (Balcar and Mendel, 1976; Löscher 1980; Ross and Graig 1981). The evidence of VPA effects on GABA uptake is thus rather inconclusive. Our adult patients exhibited changes in the transport capacity of GABA and not in the affinity for GABA. It is thus reasonable to assume that these changes were not due to VPA medication. On the other hand, in children with CAE GABA transport was increased only when they were using VPA. This may be misleading, however, being due to other differences between the patient groups. In any case, we cannot exclude the effects of VPA on GABA transport in our patients, even though the previous literature does not yield any strong support for such a conception.

5. Platelet model

The validity of our results depends on the strength of the platelet model. Platelets have previously been used for neurotransmitter studies in several neurological and psychiatric diseases for many years (Zieve and Solomon 1968, Airaksinen 1979; Mangano and Schwarcz 1981; Hambley and Jonston 1985; Ferrarese et al. 1999, 2000). It has been believed that the functions of platelets resemble those of synaptic membranes in the CNS, although it has not been known which transporters function in human platelets. Recently, Zoia and coworkers (2004) reported that human blood platelets have three glutamate transporters, EAAT1, EAAT2 and EAAT3, with kinetic parameters similar to those in the CNS. This would imply that platelets are indeed reliable peripheral markers of the glutamate behavior in the CNS. We used primers designed to recognize all glutamate transporters in platelets and the only transporter expressed was EAAT3. Our findings do not exclude the possibility that platelets also have other transporters

for glutamate, but it shows that EAAT3 is expressed most abundantly in platelets, possible being the major transporter functioning. In a similar manner we also established for the first time that human platelets express the BGT-1 transporter for GABA. This finding must be taken to show that BGT-1 has the highest expression rate in human platelets. It does not exclude the possibility that other GABA transporters may also be found in small quantities. The kinetics of GABA uptake in platelets tallies with low-affinity transport. Our present finding that BGT-1 is the transporter functioning in platelets provides a good explanation for the kinetic data. BGT-1 has been shown to transport GABA with a relatively low affinity, while all other known GABA transporters are high-affinity (Borden 1996, Gadea 2001).

After cloning both GABA and glutamate transporters from human platelets we are convinced that blood platelets can be used as a reliable model to examine changes in GABAergic and glutaminergic neurotransmission in the CNS. The changes seen in blood platelets may not be exactly the same as those in the CNS, however, since not all transporters expressed in the CNS were found in platelets.

CONCLUSIONS

According to our present studies we may draw the following inferences.

1. GABAergic neurotransmission is altered in a syndrome-specific manner in epileptic patients. The changes seem also to be related to the seizure types patients experience. Changes are seen either in the metabolism or uptake of GABA, depending on the epilepsy syndrome. Antiepileptic drugs, commonly used by our patients, do not appear to have any significant direct effects on GABA metabolism or uptake in platelets.

2. The increase in glutamate uptake in patients with localization-related seizures might be a compensatory mechanism related to the increase in plasma glutamate levels commonly seen in epileptic patients after seizures. Antiepileptics, commonly used by our patients, would not appear to have any direct effect on platelet glutamate uptake.

3. The changes in amino acids in seizure disorders are variable, in particular in the CSF. The elevated glutamate level in plasma may be characteristic of certain types of epilepsy, e.g. in idiopathic generalized epilepsies, and a sign of acute seizures.

4. Platelets can be used as a peripheral marker for amino acid uptake, since in platelets at least the BGT-1 transporter for GABA and the EAAT3 transporter for glutamate are expressed.

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