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Expression of EAAT1 reflects a possible neuroprotective function of reactive astrocytes and activated microglia following human traumatic brain injury

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Summary. Glutamate-mediated excitotoxicity is known to cause secondary brain damage following stroke and traumatic brain injury (TBI). However, clinical trials using NMDA antagonists failed. Thus, glial excitatory amino acid transporters (EAATs) might be a promising target for therapeutic intervention. Methods and Results. We examined expression of EAAT1 (GLAST) and EAAT2 (Glt-1) in 36 TBI cases by immunohistochemistry. Cortical expression of both EAATs decreased rapidly and widespread throughout the brain (in lesional, adjacent and remote areas) following TBI. In the white matter numbers of EAAT1+ parenchymal cells increased 39-fold within 24h ($p<0.001$) and remained markedly elevated till later stages in the lesion (90-fold, $p<0.01$) and in peri-lesional regions (86-fold, $p<0.01$). In contrast, EAAT2+ parenchymal cells and EAAT1+ or EAAT2+ perivascular cells did not increase significantly. Within the first days following TBI mainly activated microglia and thereafter mainly reactive astrocytes expressed EAAT1. Perivascular monocytes and foamy macrophages lacked EAAT1 immunoreactivity. We conclude that following TBI i) loss of cortical EAATs contributes to secondary brain damage, ii) glial EAAT1 expression reflects a potential neuroprotective function of microglia and astrocytes, iii) microglial EAAT1 expression is restricted to an early stage of activation, iv) blood-derived monocytes do not express EAAT1 and v) pharmacological modification of glial EAAT expression might further limit neuronal damage.

Key words: Microglial activation, Neuroprotection, Traumatic brain injury, Excitatory amino acid transporters

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Introduction

Under physiological conditions, extracellular glutamate concentration is mainly regulated through re-uptake of glutamate from the synaptic cleft via glial excitatory amino acid transporters (EAATs) (Kanner and Sharon, 1978; Wadiche et al., 1995). So far, in the human CNS five different EAATs are known. EAAT1, the human homologue of the rodent glutamate/aspartate transporter-1 (GLAST/GLAST1) and EAAT2 (homologue of glutamate transporter-1/Glt-1) are mainly expressed by glial cells, especially astrocytes, widely distributed throughout the CNS (Bonde et al., 2003; Williams et al., 2005). EAAT3 (excitatory amino acid carrier-1/EAAC1) is mainly expressed by neurons. EAAT4 and EAAT5 are restricted to the cerebellum and the retina, respectively (Simantov et al., 1999a,b). So far, no rodent homologues to EAAT4 or -5 are known.

In experimental models of ischemic (Fukamachi et al., 2001; Rao et al., 2001; Namura et al., 2002) and traumatic (Rao et al., 1998; Lopez-Redondo et al., 2000; van Landeghem et al., 2001; Krum et al., 2002) brain injury a rapid decrease of EAAT1 and EAAT2 protein levels have been found. Thereby, following TBI an early loss of astrocytic EAAT1 and -2 expression has been reported in rat brains (Rao et al., 1998). Reduced astrocytic glutamate transport capacity leads to accumulation of glutamate in the extracellular space. Subsequently, activation of glutamate receptors, in particular the NMDA receptors, leads to neuronal damage through glutamate-mediated excitotoxicity and thus contributes to secondary brain damage (Choi and Rothman, 1990; Castillo et al., 1996; Dirnagl et al., 1999; Hurtado et al., 2005). In the past several approaches have been made to reduce this glutamate-mediated neuronal damage following ischemic and traumatic brain injury by application of glutamate receptor antagonists. However, until now these approaches failed to show efficacy in clinical trials (for reviews see refs. (De Keyser et al., 1999; Ikonomidou

and Turski, 2002; Muir and Lees, 2003; Hoyte et al., 2004; Wang and Shuaib, 2005)).

These disappointing results have been explained mainly by poor quality of molecules that entered clinical trials (e.g. intolerable side effects, lack of efficacy) and poor design of clinical trials (e.g. therapeutic time window) (for reviews see refs. (De Keyser et al., 1999; Ikonomidou and Turski, 2002; Muir and Lees, 2003; Hoyte et al., 2004; Wang and Shuaib, 2005)). Recently it has been proposed that NMDA receptor antagonists failed stroke and traumatic brain injury trials in humans because a blockade of synaptic transmission mediated by NMDA receptors might hinder neuronal survival (Ikonomidou and Turski, 2002; Hoyte et al., 2004). As a consequence, glutamate-mediated neuronal damage might be limited more successfully without blockade of NMDA receptors, e.g. by action of glial glutamate transporters. Under experimental conditions both a pharmacologic upregulation as well as a blockade of EAAT1 and -2 are possible. Thus, pharmacological modification of glial EAAT activity might be a more promising approach to reduce glutamate-mediated neuronal damage than blockade of NMDA receptors.

So far, no data on the expression of EAAT1 or -2 by activated microglia or astrocytes following TBI in human brains have been reported. We therefore investigated the expression of the main glial EAATs, EAAT1 and EAAT2, in human traumatic brain lesions to define a pathophysiological basis for possible future therapeutic approaches.

Material and methods

Cases

We investigated autoptic brain specimens derived from 36 patients from an updated series of cases who died after varying survival times following closed TBI reported previously (Beschorner et al., 2002). For clinical and autopsy data of cases see table 1. In addition to patient data, hematoxylin-eosin (HE), luxol fast blue (LFB) and iron (Fe) staining was used for evaluation of the typical histological features defined as standard indicators of trauma age (Graham et al., 2002). In all cases histopathological stages of the brain lesion corresponded to clinical data. Tissue samples from the lesion were investigated in all cases. Additionally, samples from peri-lesional (adjacent) and remote areas were investigated if available. Samples from corresponding brain areas from nine cases out of a previously published normal brain bank served as controls (Mittelbronn et al., 2001).

Immunohistochemistry and double labeling experiments

Following re-hydration, formalin-fixed paraffin-embedded tissue sections were boiled in a microwave oven (600-W) four times for five minutes in citrate buffer (pH 6.0). Endogenous peroxidase was inhibited with 1% H_2O_2 in methanol for 15 minutes and 10% normal porcine serum (Biochrom, Berlin, Germany) was applied to block non-specific binding of immunoglobulins. In all cases immunohistochemistry for EAAT1 (NCL-EAAT1, Novocastra, clone 10D4, monoclonal, IgG1, dilution 1:100) and EAAT2 (NCL-EAAT2, Novocastra, clone 1H8, monoclonal, IgG2a, dilution 1:100) was performed. The primary antibody (EAAT1 or EAAT2) was applied overnight at 4°C. Antibody binding was visualized with a biotinylated secondary antibody (rabbit-anti-mouse), streptavidin and biotinylated horseradish peroxidase (HRP) complex (StreptABComplex/HRP; Dako GmbH, Glostrup, Denmark) and diaminobenzidine (DAB; Dako) as chromogen. Sections were counterstained with Mayer's hemalaun. Negative controls consisted of sections incubated in the absence of the primary antibody. Furthermore, for EAAT1 and EAAT2 isotype controls were also done (IgG1 and IgG2a, DakoCytomation, Glostrup, Denmark).

Additionally, in selected cases double immunolabeling experiments were performed on an automated immunohistochemistry slide staining system (BenchMark®, Ventana Medical Systems, Tucson, Az, USA) using 'Dual Staining i-View DAB/Enhanced Ventana Red'. Thereby first EAAT1 was detected using a peroxidase detection kit (Ventana, i-View DAB, ref# 760-091) revealing a brown reaction product by DAB. Thereafter, antibodies against glial fibrillary acid protein (GFAP, Novocastra, Newcastle, UK, polyclonal, dilution 1:500) or against the monocytic antigen CD68 (DakoCytomation, Glostrup, Denmark, monoclonal, dilution 1:100) were applied and visualized using an alkaline phosphatase kit (Ventana, Enhanced Ventana Red, ref#760-031), revealing a red reaction product by Ventana-Red. In double-labeling experiments, counterstaining with Mayer's hemalaun was performed after visualization of the EAAT1 (Figs. 3E,F). Furthermore, in selected cases double labellings were also performed using a manual staining protocol. Thereby, first expression of EAAT1 was visualized as described above. Then, after additional irradiation in a microwave oven, antibodies against CD68 and GFAP were applied. Visualization was achieved using biotinylated secondary antibodies, alkaline phosphatase-conjugated ABC complex and Fast-Blue BB salt (Sigma, Deisenhofen, Germany) as chromogen revealing a blue reaction product. Thus, following manual double labelings no counterstaining of nuclei was done (Figs. 3C,D).

Evaluation and statistical analysis

Evaluation of EAAT1 immunolabelings was done separately in the core of the lesion, in the peri-lesional tissue adjacent to the traumatic core and (if available) in areas remote from the lesion.

For statistical analysis cases were clumped into four groups with increasing survival times post trauma: up to 24 h (n=20), 1–7 days (n=6), 1-9 weeks (n=8), more than 9 weeks (n=2; compare table 1 and Fig 4).

Cortical EAAT1 and EAAT2 immunoreactivity was evaluated using a 4 scaled semi-quantitative score (0-3) reflecting distribution and intensity of cortical EAAT expression in comparison to corresponding regions from control brains: (3), intense, diffuse or patchy immunostaining pattern equal to controls; (2), clearly decreased but still easily visible (diffuse or patchy) staining; (1), markedly reduced staining with only minor focal residual EAAT expression usually restricted to a single cortical layer; (0), no staining.

For evaluation of EAAT1 and EAAT2 immunoreactivity in white matter regions, the numbers of immunoreactive 'parenchymal cells' were counted in ten high power fields (HPF; x 400 magnification with an eyepiece grid representing 0.0625 mm^2 each). In the same area numbers of immunostained 'perivascular cells' (cells in perivascular spaces) were counted per ten blood vessels. According to an expected location of EAATs at the cell membrane (Duan et al., 1999; Voutsinos-Porche et al., 2003; Lopez-Bayghen and Ortega, 2004) cells with an obvious cytoplasm (e.g. reactive astrocytes, macrophages) were only considered positive if they showed a membranous immunostaining.

Statistical analysis

The scores for cortical EAAT expression obtained from the lesion, adjacent (peri-lesional) areas and remote areas, respectively, were compared to controls. We used a global Kruskal-Wallis test to analyse the overall difference between groups. A significant global test with a p-value <0.05 was followed by a post-hoc analysis

Table 1. Clinical and autopsy data from cases following traumatic brain injury.

Case	Age	Sex	Time p.i.	Trauma	Lesion	Cause of death	Additional clinical and autoptical data
1	64	m	0 h	traffic	frontoparietal l/r	disruption of brain stem	CF, BSF, enterorrhexis
2	57	m	0 h	traffic	frontotemporal l/r	polytrauma	CF, BSF, SAH, hepatorrhexis
3	49	m	1 h	fall	frontobasal-parietal	asystole	DAI
4	16	m	1 h	traffic	frontotemporal l/r	severe brain edema	CF, SAH, polytrauma
5	31	m	1.25 h	percussion	frontobasal r	severe brain edema	BSF, DAI, intraventricular bleeding, Res.
6	60	m	2.5 h	traffic	temporobasal l/r	hemorrhagic shock	BSF, SAH, polytrauma
7	36	m	3 h	traffic	frontotemporoparietal l/r	severe brain edema	CF, BSF, SAH, polytrauma, fat embolism
8	93	f	5 h	traffic	occipital l	polytrauma	CF
9	20	m	5.2 h	fall	temporobasal l	polytrauma	vertebral fracture, concealed hemorrhage, herniation
10	27	m	5.5 h	fall	parietooccipital r	severe TBI	air embolism, Res.
11	63	f	5.7 h	traffic	frontoparietal l/r	polytrauma	BSF, SDH, SAH, hemorrhagic pleurorrhea
12	27	f	< 6 h	traffic	frontal l/r	herniation	SDH, Trep., DAI
13	43	m	< 6 h	fall	frontotemporal l/r	herniation	Chronic alcoholism
14	63	m	7 h	fall	frontal pole l	severe brain edema	EDH
15	46	m	12 h	fall	frontobasal r	severe brain edema	CF, BSF, EDH, tonsillar herniation
16	42	f	12 h	traffic	frontal l	hemorrhagic shock	CF, BSF, polytrauma, splenic rupture, air embolism
17	29	m	12 h	traffic	temporal r	central regulation failure	severe injuries of both legs, hemorrhagic shock
18	28	m	14 h	traffic	frontotemporal l	hemorrhagic shock	SAH, DAI, anoxic encephalopathy
19	44	m	20 h	fall	frontobasal l	severe TBI	CF, BSF, EDH, SDH, air embolism
20	52	m	20 h	fall	frontal r	herniation	SDH
21	87	f	36 h	traffic	frontal pole l/r	severe TBI	CF, BSF, SDH,
22	64	m	48 h	fall	parietotemporal r	severe brain edema	CF, BSF, SAH, DAI, Trep., liver cirrhosis
23	60	m	4 days	fall	temporal l	severe TBI	CF, severe intracerebral hemorrhage
24	84	f	4 days	traffic	frontal callosal	polytrauma	Diffuse focal hemorrhages in white matter
25	58	m	6 days	fall	temporobasal l	herniation	CF, EDH, SDH
26	59	m	7 days	traffic	temporal r	herniation	Polytrauma, BSF
27	55	m	8 days	traffic	frontotemporal l/r	anoxic encephalopathy	CF, BSF
28	47	m	9 days	fall	frontotemp l/r	severe TBI	CF, BSF, EDH, SDH, SAH, Trep., l PCA infarction, organ donor
29	41	m	10 days	traffic	frontotemporal l/r	contusion of brain stem	CF, BSF, DAI, multiple rip fractures, wet lung
30	38	m	12 days	fall	temporal l/r, frontal l	herniation	EDH, SDH, DAI
31	83	m	14 days	traffic	temporobasal r	pneumonia	SDH, Trep., hematoma 48-72 h, CLL
32	80	f	15 days	traffic	temporobasal l/r	herniation	CF, DAI
33	18	f	16 days	traffic	frontotemporal l/r	multiple-organ failure	polytrauma, fracture of cervical vertebral spine, SAH, Trep., Res.
34	19	m	27 days	traffic	frontotemporal l/r	severe brain edema	BSF, EDH, SAH, Trep., resection of left temporal lobe
35	56	f	6 months	traffic	frontobasal l	pneumonia	SDH, CF, ischemia, hemiplegia
36	29	m	60 months	fall	frontobasal l/r	pulmonary embolism	Infantil brain disease of uncertain origin

ARDS: adult respiratory distress syndrome; BSF: basal skull fracture; CF: calvarial fracture; CLL: chronic lymphocytic leukemia; DAI: diffuse axonal injury; EDH: epidural hemorrhage; l/r: left and right; l: left; n.i.: no information; PCA: posterior cerebral artery; p.i.: post injury; Preg.: pregnancy; r: right; Res.: resuscitation; SAH: subarachnoidal hemorrhage; SDH: subdural hemorrhage; TBI: traumatic brain injury; Trep.: trepanation

using the Wilcoxon test comparing each group with the respective control group. Post-hoc p-values were corrected for multiple comparisons using the Bonferroni-Holm correction. Analysis of the number of immunoreactive cells in the white matter (parenchymal cells and perivascular cells, respectively) showed non-normality despite log-transformation for the cell counts in the different groups as assessed by the Shapiro-Wilk-W-test. Additionally, variance across the groups was unequal. Thus, statistical analysis of these cell counts was done analogous to the analysis of the cortical scores. We considered a Bonferroni-Holm corrected p-value of 0.05 to be significant. Fold changes were calculated on the basis of the respective group means in comparison to controls.

Results

EAAT1 and EAAT2 in control brains

Both EAAT1 and EAAT2 are intensively expressed in cortical areas, thereby showing either a diffuse or a patchy immunostaining in all cortical layers (Fig. 1A,B,D,E). Generally, in the white matter no immunoreactivity for EAAT1 (Fig. 1A,C) or EAAT2 (Fig. 1D,F) was found. Only in two different control brains single parenchymal glial cells expressing EAAT1 (1/9 cases) or EAAT2 (1/9 cases) were found in the white matter, respectively. In perivascular spaces only single EAAT1+ cells were detected in one case. No EAAT2+ perivascular cells were found.

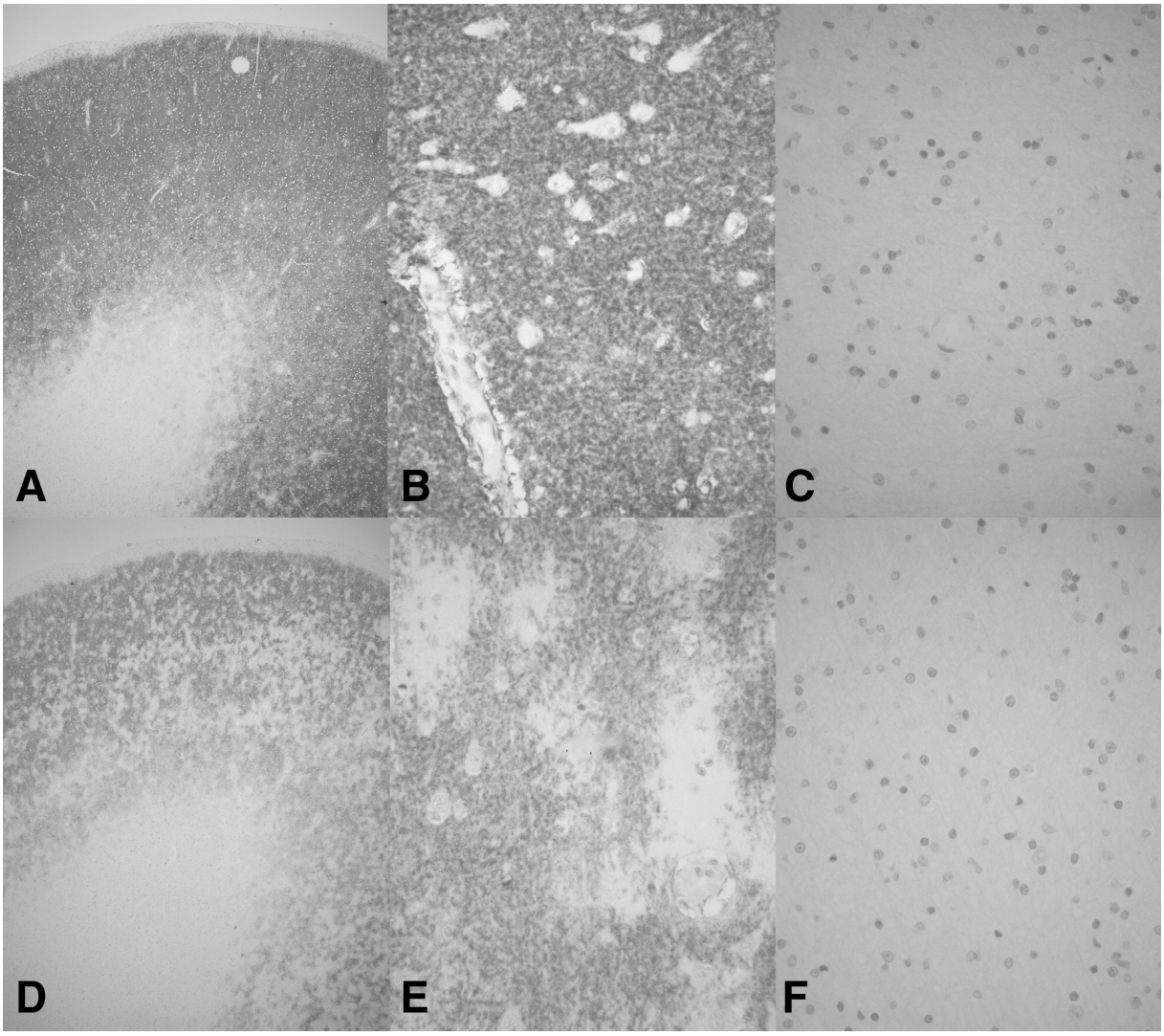


Fig. 1. Expression of EAAT1 and -2 in normal human brains. In controls a strong diffuse expression of EAAT1 is found in the frontal cortex **(A, B)**. In the white matter no EAAT1+ cells are found **(C)**. Similarly, EAAT2 is strongly expressed in the cortex showing a diffuse or patchy staining pattern **(D, E)** but is absent in the white matter **(F)**. A, D, x 25; B, C, E, F, x 400

EAAT1 following TBI

Cortical expression of EAAT1 and numbers of parenchymal EAAT1+ cells in the white matter are shown in figures 2A,B and 4A,C, respectively.

EAAT1 in grey matter following TBI

Reduction in cortical EAAT1 expression occurs rapidly, widespread throughout the brain (i.e. in lesional, adjacent and remote areas) and lasts long. In the grey matter reduction of EAAT1 immunoreactivity is obvious within less than one hour post trauma, reaches statistical significance within 24 hours in lesional (reduced on average to 0.5-fold level of controls; $p<0.001$) and perilesional (0.6-fold; $p<0.01$) as well as in remote regions (0.6-fold; $p<0.01$). Cortical expression of EAAT1 remains markedly reduced in all regions till later stages. After 1-9 weeks following TBI cortical EAAT1 expression was reduced 0.6-fold at the lesion ($p<0.01$), 0.7-fold in adjacent regions ($p<0.01$) and 0.5-fold in remote areas ($p<0.05$).

EAAT1 in white matter cells

Parenchymal EAAT1+ cells appeared within 2.5 hours and their mean numbers increased 39-fold at the lesion within 24 hours ($p<0.05$), 34-fold in adjacent

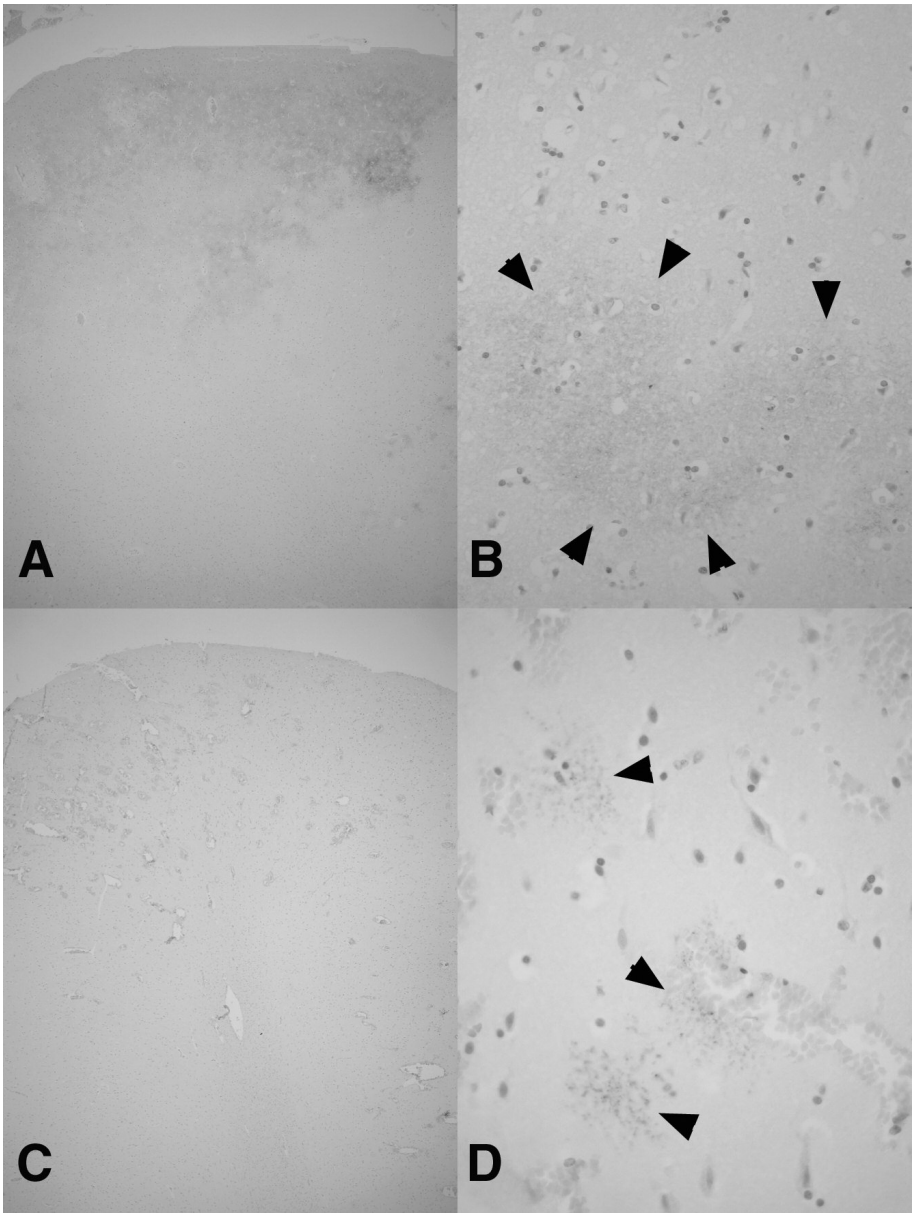
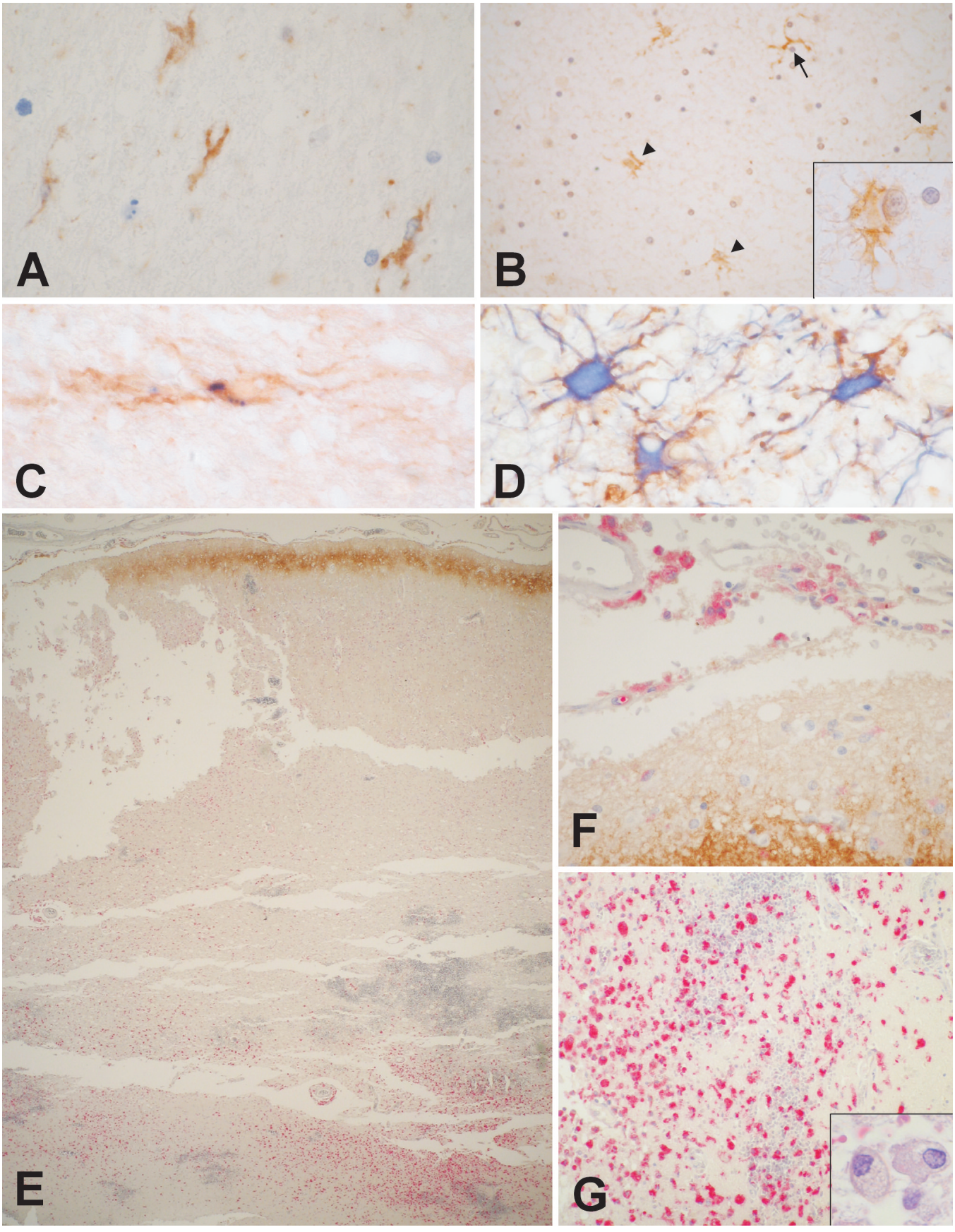


Fig. 2. Cortical expression of EAAT1 **(A, B)** and EAAT2 **(C, D)** decreases rapidly following TBI. Only focally weak to moderate immunoreactivity is preserved (arrows in **B, D**). A, C, x 25; B, D, x 400


A
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regions ($p<0.05$) and 7-fold in remote areas ($p>0.05$).

At the lesion numbers of EAAT1+cells reached maximum after 1-7 days (134-fold, $p<0.01$) and remained increased after 1-9 weeks (90-fold, $p<0.01$). In later stages (more than nine weeks) numbers of EAAT1+ cells remained increased but did not reach statistical

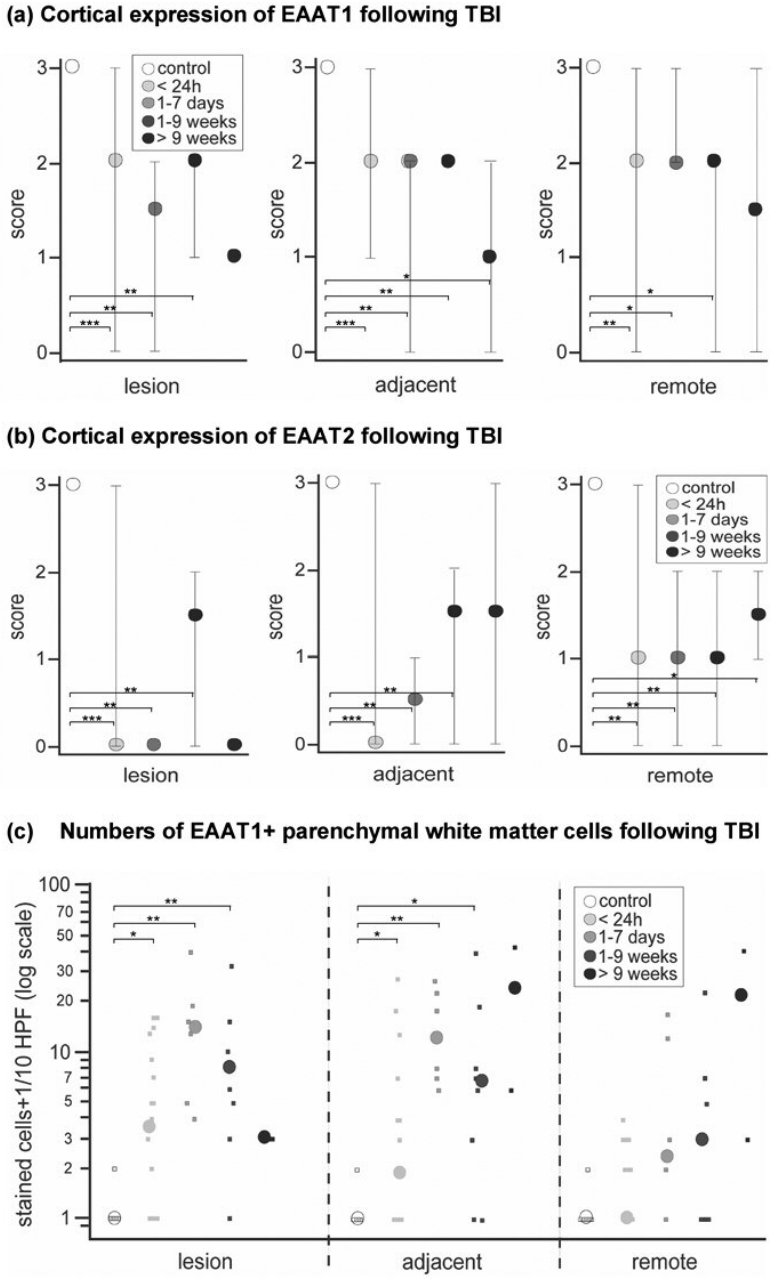

Fig. 4. Shows data obtained from controls and from TBI cases clustered into groups with increasing survival times. In **(a)** and **(b)** cortical expression of EAAT1 and EAAT2 following TBI is shown according to a semi-quantitative score in comparison to controls, respectively. In **(c)** numbers of EAAT1+ parenchymal cells in the white matter following TBI are shown. Significance at an alpha level of <0.05 (*), <0.01 (**) and <0.001 (***) is illustrated.

Fig. 3. Expression of EAAT1 by parenchymal white matter cells following TBI. Within the first days following TBI EAAT1+ cells in the white matter morphologically resemble activated ramified microglia **(A)**. In later stages, beside cells with typical microglial morphology (arrow, **B**) also astrocytic-like cells show a membranous expression of EAAT1 (arrow heads and inset in B). Note membraneous immunoreactivity (inset in B). Double-immunolabelings using antibodies against CD68 (blue, **C**) and GFAP (blue, **D**) show cellular coexpression with EAAT1 (brown). In subacute stages double-immunostaining shows residual EAAT1 expression (brown) in the second cortical layer but not in areas with accumulation of CD68+ (red) activated microglia/macrophages (**E**). Higher magnifications from (**E**) show CD68+ macrophages/activated microglia (red) in leptomeninges (**F**) and in subcortical white matter (**G**) not to coexpress EAAT1 (brown). Inset in (**G**) shows histology of foamy macrophages in the white matter (HE). A, C, D and insets in B, E, x 1000; B, E, x 400

significance (18-fold, $p>0.05$).

In adjacent areas numbers of EAAT1+ cells also further increases after 1-7 days (125-fold, $p<0.05$).
In remote areas counts for EAAT1+ parenchymal cells also increased on average (48-fold after 1-9 weeks, 189-fold after >9 weeks) but did not reach formal statistical significance, since only the trend reached statistical significance ($p=0.015$) but not group comparison ($p=0.102$).

EAAT1 in perivascular cells

In perivascular spaces only very occasionally single EAAT1+ cells were found, not reaching statistical significance at any time-point or in any of the examined regions (not shown).

EAAT2 following TBI

Cortical expression of EAAT2 following TBI is shown in figures 2C,D and 4B.

Similarly to EAAT1, the expression of EAAT2 in cortical areas decreases rapidly and widespread throughout the brain and remains significantly decreased till several weeks post trauma. Obvious reduction of cortical EAAT2 immunoreactivity starts within less than one hour following TBI.

In all regions examined parenchymal cells expressing EAAT2 were found only in minor numbers in few cases (not shown).

Perivascular cells showing immunoreactivity for EAAT2 were exceptionally rare (not shown). Thus, there was no significant change in average numbers of immunoreactive parenchymal cells or perivascular cells when compared to controls.

Cellular sources of EAAT1 expression following TBI (double-labeling experiments)

In earlier stages EAAT1-positive cells morphologically mainly resembeled ramified microglia (Fig. 3A-C). In later stages showing reactive astrogliosis and infiltration by phagocytizing macrophages reactive astrocytes are the main cellular source of EAAT1 expression (Fig. 3D) while CD68+ foamy macrophages lack EAAT1-immunoreactivity (Fig. 3F,G).

Discussion

Glutamate-mediated excitotoxicity is well known to occur rapidly after brain injury of various types and to contribute to secondary neuronal damage. Numerous studies have demonstrated a reduction of astrocytic expression of EAAT1 (GLAST) and EAAT2 (Glt-1) after various types of brain injury (Anderson, 1991; Torp et al., 1995; Ginsberg, 1996; Knecht et al., 1997), including TBI (Rao et al., 1998; van Landeghem et al., 2001; Ikematsu et al., 2002). These data lead to the concept that glial glutamate transporter expression is dependent upon glutamatergic innervation and undisturbed metabolic parameters and that decreases in glutamate transport result in delayed neuronal death (Krum et al., 2002).

In experimental studies detrimental effects of elevated extracellular glutamate concentrations can be reduced by blockade of glutamate binding to glutamate N-methyl-D-aspartat (NMDA) receptors. Thereupon, several approaches with the aim of preventing glutamate-mediated excitotoxicity using NMDA receptor antagonists entered clinical trials. Yet, so far, NMDA receptor antagonists have failed clinical trials for ischemic stroke and TBI suggesting that a blockade of the synaptic transmission mediated by NMDA receptors hinders neuronal survival (Ikonomidou and Turski, 2002). Another basic approach to prevent glutamate-mediated secondary brain damage is to reduce extracellular glutamate concentration in the synaptic cleft via re-uptake of glutamate by excitatory amino acid transporters (EAATs). In the human brain especially the high affinity glutamate transporters EAAT1 (GLAST) and EAAT2 (Glt-1) are potential candidates for regulating extracellular glutamate concentration (Simantov et al., 1999a,b; Bonde et al., 2003; Williams et al., 2005). Glial expression of EAAT1 can be modulated pharmacologically and pharmacologic up-regulation of EAAT1 is associated with reduced infarct volume and improved neurological outcome following middle cerebral artery occlusion in rats (Matsuura et al., 2002; Mori et al., 2004). Similarly, pharmacologic modification of EAAT2 expression has been reported (Robelet et al., 2004; Eisenstein, 2005; Hurtado et al., 2005; Rothstein et al., 2005).

In rodent cell cultures an early upregulation of EAAT1 and -2 by reactive astrocytes and activated microglial cells has been reported (Swanson et al., 1997; Lopez-Redondo et al., 2000; Nakajima et al., 2001; Persson et al., 2005; Pawlak et al., 2005). In models of rat TBI an upregulation of EAAT1 and -2 by activated astrocytes and microglia has also been found in vivo (Lopez-Redondo et al., 2000; van Landeghem et al., 2001).

Expression of EAAT1 and -2 by activated microglial cells reflects a possible neuroprotective function which has been reported in several in vitro and in vivo studies such as in rat microglial cell cultures (Lopez-Redondo et al., 2000; Nakajima et al., 2001; Persson et al., 2005), SIVmac251-infected macaques (Chretien et al., 2002), human HIV encephalitis (Gras et al., 2003; Vallat-Decouvelaere et al., 2003), human prion disease (Chretien et al., 2004) and experimental TBI (Lopez-Redondo et al., 2000; van Landeghem et al., 2001). Further studies focused on expression of EAATs in the grey matter following rat TBI, rat spinal cord injury and in human TBI but did not investigate whether reactive astrocytes or activated microglia express EAAT1 or -2 (Rao et al., 1998; Ikematsu et al., 2002; Vera-Portocarrero et al., 2002).

Several in vivo and in vitro studies reported an upregulation and increased biological activity of EAAT1 and -2 by several drugs/molecules such as MS-153

(Shimada et al., 1999), dibutyryl cyclic (dbc)AMP and pituitary adenylate cyclase-activating polypeptide (PACAP) (Rozyczka et al., 2004), citicoline (Hurtado et al., 2005), arundic acid (ONO-2506) (Mori et al., 2004) and beta-lactam antibiotics (e.g. ceftriaxone) (Rothstein et al., 2005). Furthermore, beneficial effects such as a reduction of infarct volume and an improved neurological outcome could be achieved in experimental stroke models (Umemura et al., 1996; Mori et al., 2004; Hurtado et al., 2005; Rothstein et al., 2005). In experimental spinal cord injury or TBI PACAP and citicoline have been reported to reduce apoptotic cell death, brain oedema and numbers of damaged axons (axonal injury) and to improve neurological outcome (Baskaya et al., 2000; Farkas et al., 2004; Cakir et al., 2005; Chen and Tzeng, 2005). The functionally relevant role of EAATs regarding neuronal activity and survival has been shown in knockout mice and by application of antisense oligonucleotids leading to reduced levels of EAAT1 and -2 (Rothstein et al., 1993, 1996; Tanaka et al., 1997; Niederberger et al., 2003). Thus, under experimental conditions, a pharmacological upregulation as well as a blockade of EAAT1 and -2 are possible.

The expression patterns of EAAT1 and -2 in our controls are in accordance with previous reports (Ikematsu et al., 2001; Banner et al., 2002; Chretien et al., 2004; Williams et al., 2005).

Following TBI, a rapid and widespread decrease of cortical EAAT1 and EAAT2 expression occurs, reflecting early risk of neurons for excitotoxicity. Thereby, the widespread and long lasting decrease of both EAATs even in areas remote to the lesion potentially reflects increased vulnerability to excitotoxicity neither to be restricted to lesional and peri-lesional (adjacent) areas nor to be confined to early stages following TBI.

Whether early loss of cortical EAAT1 and -2 expression following TBI is due to a break down in protein synthesis, due to a loss of EAAT expressing cells (i.e. necrosis or apoptosis of astrocytes) or the result of a combined effect requires functional in vivo and in vitro studies. As glutamate transporters have a short half-life (Palacin et al., 1998; Fournier et al., 2004) and necrotic and apoptotic-like cell death occurs rapidly after TBI (Kaya et al., 1999; Clausen et al., 2004) it is likely a combination of both effects.

The rapid upregulation of EAAT1 by microglia and astrocytes in the white matter throughout the brain corresponds to the widespread loss of cortical EAAT expression and potentially reflects a compensatory neuroprotective role of activated microglia and astrocytes. However, as the main effect of excitotoxicity happens within minutes to a few hours following brain injury (Dirnagl et al., 1999) the glial upregulation of EAAT1 is relatively delayed and lags behind loss of cortical expression. Furthermore, glial upregulation of EAAT1 mainly takes place at some distance to vulnerable neurons, namely in the white matter. Thus, we assume that a compensatory endogenous attempt of microglia and astrocytes to prevent glutamate-mediated neuronal damage is insufficient. Attempts to further increase glial expression of EAAT1 and -2 might therefore limit secondary brain damage and improve clinical outcome following TBI.

In experimental stroke models a rapid and excessive increase in extracellular glutamate levels occurs, exceeding normal concentrations 10-100 times. However, this increase is only seen for 10-30 min after injury (Benveniste et al., 1984). In contrast, microdialysis of human brain tissue after TBI showed sustained (days to weeks) but minor (0.5-1 times) increase in glutamate concentrations (Bondoli et al., 1981; Persson and Hillered, 1992; Baker et al., 1993). Unfortunately it is impossible to measure extracellular glutamate concentration in humans immediately after onset of brain injury. The prolonged increase in glutamate concentrations in human traumatic brain lesions has been considered neurotoxic and interpreted as an opportunity for a delayed therapy with NMDA antagonists. However, this theory was doubted as such mild elevations in glutamate concentrations may reflect a self-defense mechanism of the injured brain, which may promote survival of endangered neurons and facilitate tissue repair (Ikonomidou and Turski, 2002).

Furthermore, it has been shown that delayed treatment with NMDA antagonists (i.e start of administration 1-7 h after trauma) harmed neurons subjected to traumatic brain injury (Ikonomidou et al., 2000) and suppresses neurogenesis triggered by focal cerebral ischemia in the hippocampus (Arvidsson et al., 2001). These data lead to the hypothesis that glutamate kills neurons immediately after the injury but starts to facilitate repair shortly thereafter. In contrast to its excitatory effect, repair mediated by glutamate appears to be long lasting (Ikonomidou and Turski, 2002).

Interestingly, several studies reported evidence for a time-dependent role of EAAT1 and -2 following ischemia. Thereby, the function of EAATs switched after the early phase to reversed transport (i. e. glutamate release) resulting in increasing extracellular glutamate levels (Phillis et al., 2000; Rossi et al., 2000; Bonde et al., 2003; Mitani and Tanaka, 2003).

Thus, future therapeutic approaches should not limit postsynaptic glutamate action through glutamate receptor antagonists (NMDA blockers) but may try to regulate extracellular glutamate concentrations by modification of the glial glutamate transporters. However, if the functional role of EAAT1 and -2 in human brain lesions is time-dependent (i.e. switching from glutamate uptake to glutamate release) and consequential conclusions for potential therapeutic strategies (time window, pharmacologic upregulation or blockade of EAATs) require further functional studies, e.g. on human astrocytic and microglial cell cultures.

In the literature variable data on glial expression of EAAT1 and -2 are reported. Thereby different data are reported from various in vitro and in vivo studies as well as in human brains under different pathologic conditions. For instance, microglia was reported to express EAAT1 in different experimental (Rimaniol et al., 2000; van

Landeghem et al., 2001) and human (Vallat-Decouvelaere et al., 2003; Chretien et al., 2004) studies while others found no or only minor EAAT1 expression in experimental (Swanson et al., 1997; Nakajima et al., 2001) or human (Werner et al., 2001) studies. Likewise, some studies reported astrocytes to express EAAT1 (Swanson et al., 1997; Nakajima et al., 2001; van Landeghem et al., 2001; Pawlak et al., 2005) while others found astrocytes to lack EAAT1 (Werner et al., 2001; Vallat-Decouvelaere et al., 2003; Chretien et al., 2004). Furthermore, variable reports on EAAT2 expression of activated microglial cells and astrocytes exist (Swanson et al., 1997; Lopez-Redondo et al., 2000; Nakajima et al., 2001; van Landeghem et al., 2001; Werner et al., 2001; Chretien et al., 2002; Hurtado et al., 2005; Pawlak et al., 2005; Persson et al., 2005). The multitude of diverse data regarding expression of EAATs by microglia and astrocytes points to both differences between species as well as differences among variable diseases or experimental conditions.

In the present study we found resting astrocytes and microglia in the white matter to lack EAAT1 immunoreactivity. Following human TBI, activated astrocytes and microglia express EAAT1. Thereby, EAAT1 immunoreactivity on microglial cells was restricted to an early or intermediate state of activation (i.e. microglia with ramified to ameboid morphology) and got lost prior to differentiation into foamy macrophages. Additionally, monocytic cells in perivascular spaces reflecting blood-derived infiltrating monocytes lacked EAAT1 expression. Thus, microglial EAAT1 expression following human TBI is confined to an early/intermediate stage of activation and may distinguish activated resident microglia from infiltrating blood-derived monocytes.

In contrast to several experimental studies, in our cases glial white matter cells almost exclusively lack EAAT2 expression (Swanson et al., 1997; van Landeghem et al., 2001; Pawlak et al., 2005).

Thus, endogenous upregulation of EAAT2 on glial white matter cells is much less than upregulation of EAAT1. This is of special importance as EAAT2 is functionally the most active subtype of glutamate transporters accounting for more than 90% of glutamate reuptake in the forebrain (for review see ref. (Robinson, 1999)). Whether the lack in EAAT2 upregulation by glial cells reflects an even larger hidden neuroprotective potential for a pharmacologic modulation of EAAT2 at different time points following TBI or points out EAAT1 to be the most promising candidate for a therapeutic intervention requires further studies.

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