Université de Montréal

LES MÉCANISMES EXCITOTOXIQUES ET LE RÔLE DE TRANSPORTEURS DE GLUTAMATE DANS LA PHYSIOPATHOLOGIE DES TRAUMATISMES CRÂNIENS

EXCITOTOXIC MECHANISMS AND THE ROLE OF GLUTAMATE TRANSPORTERS IN THE PATHOPHYSIOLOGY OF TRAUMATIC BRAIN INJURY

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ABSTRACT

Traumatic brain injury (TBI) is one of the leading causes of death and disability in industrialized countries. The neurological consequences of such an injury are complex and may impair any of sensory, motor, or autonomic functions. Primary injuries occur at the moment of injury and are irreversible, whereas secondary injuries can evolve from the initial primary injury, and are often the focal targets of therapeutic interventions.

Glutamate is a major excitatory neurotransmitter in the brain. Over-stimulation of postsynaptic glutamate receptors due to increased extracellular glutamate levels could result in neuronal loss from excitotoxicity, and constitutes one of the major secondary injuries following TBI where a rise in brain glutamate levels has been documented both in humans and animals.

This thesis investigates excitotoxic mechanisms and the role of glutamate transporters in the pathophysiology of TBI. A well-characterized rat model of TBI, involving lateral parasagittal fluid-percussion injury (FPI), is utilized to investigate the events involved in the early increase of extracellular glutamate following TBI.

In Article 1, it is demonstrated that the downregulation of brain glutamate transporter GLT-1v protein levels occurs in the injured cerebral cortex and is correlated closely with neuronal loss seen in the same injury area. Such loss in GLT-1v may result in impaired glutamate uptake, giving rise to excitotoxic injury following FPI.

In the second article, it is demonstrated that a transient rise of complexin I and II protein levels is detected in the injured cerebral cortex that can lead to the increased neurotransmitter release from presynaptic neurons. Changes in complexin levels were completely blocked by *N*-acetylcysteine (NAC) administered 5 minutes following trauma, suggesting an involvement of oxidative stress. Neuronal loss was also reduced in the injured hemisphere with post-TBI NAC treatment.

In the third article, an increase in EAAT4 protein levels was detected that may represent a compensatory mechanism to counteract the loss in GLT-1v protein levels

following TBI. This EAAT4 increase observed following injury was primarily localized to cells morphologically resembling astrocytes.

Altogether, these results suggest a complex process of excitotoxic injury is involved in the pathophysiology of FPI.

Key words: Traumatic brain injury, fluid-percussion injury, glutamate, glutamate transporter, GLT-1v, EAAT4, neurotransmitter release, complexin I, complexin II, excitotoxicity, antioxidant.

RÉSUMÉ

Les traumatismes crâniens sont l'une des causes majeures de décès et de dysfonctions physiques chez les adultes dans les pays industrialisés. Les conséquences neurologiques d'un traumatisme crânien sont complexes et affectent les fonctions motrices, la sensation et la mémoire. Les traumatismes sont classifiés dans deux catégories (primaire et secondaire) selon le type et l'aspect de la lésion. Les lésions primaires se manifestent au moment du traumatisme et sont irréversibles. Par contre, la progression des lésions primaires peut entraîner des lésions secondaires qui nécessiteront des interventions thérapeutiques.

Le glutamate est un neurotransmetteur excitateur majeur dans le cerveau. Des niveaux élevés de glutamate extracellulaire peuvent entraîner une surexcitation des neurones post-synaptiques chez les patients atteints de traumatismes crâniens, causant une dépolarisation des neurones suivi de l'élévation de la concentration de calcium intracellulaire et de l'activation des mécanismes apoptotique ou nécrotique, causant la mort des neurones par un phénomène d'excitotoxicité. Ceci constitue une des conséquences secondaires majeures des traumatismes crâniens tel que démontrées par l'élévation de la concentration cérébrale de glutamate observée chez les patients ainsi que dans les modèles animaux de traumatismes crâniens.

La présente thèse vise à explorer les mécanismes excitotoxiques et le rôle des transporteurs de glutamate dans la physiopathologie des traumatismes crâniens. Un modèle de traumatismes crâniens latéro-parasagittale (traumatismes par percussion hydraulique) a été utilisé pour étudier les mécanismes impliqués dans l'augmentation du glutamate extracellulaire chez le rat.

Le chapitre 2.1 (Article 1) démontre une baisse des niveaux d'expression du transporteur du glutamate GLT-1v dans la région cérébrale traumatisée qui est en corrélation avec la perte neuronale. La diminution des niveaux de GLT-1v entraîne une perte de la capacité de re-capture du glutamate dans la région affectée résultant en une augmentation de la susceptibilité des neurones à l'excitotoxicité.

Dans le chapitre 2.2 (Article 2) une augmentation des niveaux d'expression des protéines Complexin I et Complexin II est observée dans le cortex cérébral

traumatisé, ce qui suggère une propension pour la relâche des neurotransmetteurs pré-synaptiques dans cette région. Cette augmentation est entièrement bloquée par l'administration de l'anti-oxydant *N*-acetylcystéine (NAC), ce qui démontre un rôle du stress oxydatif. Le fait que la perte des neurones soit réduite dans l'hémisphère touché après l'administration de NAC suggère que l'augmentation des Complexins peut contribuer à la physiopathologie des traumatismes crâniens.

Dans le chapitre 2.3 (Article 3), nous avons observé une augmentation des niveaux d'expression du transporteur du glutamate EAAT4 consécutif aux traumatismes suggérant qu'un mécanisme prend place pour compenser la perte des niveaux d'expression de GLT-1v. Cette augmentation de EAAT4 a lieu dans des cellules dont la morphologie est caractéristique de l'astrocyte.

En conclusion, les résultats présentés dans cette thèse démontrent que des mécanismes d'excitotoxicités complexes sont impliqués dans la physiopathologie des traumatismes crâniens.

Mots clés: traumatismes crâniens, percussion hydraulique, glutamate, transporteurs du glutamate, GLT-1v, EAAT4, neurotransmetteurs, complexin I, complexin II, excitotoxicité, anti-oxydant.

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ABREVIATIONS

ALS	Amyotrophic lateral sclerosis
ALS-PDC	Amyotrophic lateral sclerosis-parkinsonism dementia comlex
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
SNAP	Soluble *N*-ethylmaleimide-sensitive factor-attachment protein
BBB	Blood-brain barrier
CBF	Cerebral blood flow
CCII	Controlled cortical impact injury
CCP	Craniocerebral pressure
CMR_{glu}	Cerebral metabolic rate for glucose
$CMRO_2$	Cerebral metabolic rate for oxygen
CPP	Cerebral perfusion pressure
Cpx	Complexin
CT	Computerized tomography
DAB	3,3'-diaminobenzidine
EAA	Excitatory amino acid
EAAC	Excitatory amino acid carrier
EAAT	Excitatory amino acid transporter
ECL	Enhanced chemiluminescence
EEG	Electroencephalogram
FPI	Fluid-percussion injury
GABA	Gamma-aminobutyric acid
GCS	Glasgow coma score
GFAP	Glial fibrillary acidic protein
Gl	Granule cell layer
GLAST	Glutamate-aspartate transporter
GLT	Glutamate transporter
Glu	Glutamate
GS	Glutamine synthetase

ICP	Intracranial pressure
IgG	Immunoglobulin G
Hc	Hippocampus
HRP	Horse radish peroxidase
IC	Injured cortex
i.p.	Intraperitoneal
MCAO	Middle-cerebral artery occlusion
mGluR	Metabotropic glutamate receptor
Ml	Molecular layer
NAC	*N*-acetylcysteine
NIH	National institute of health
NMDA	*N*-methyl-*D*-aspartate
NT	Nitrotyrosine
OEF	Oxygen extraction fraction
PaO_2	Blood oxygen pressure
PBS	Phosphate buffered saline
PDZ	PSD-95/SAP90, discs large and Zona occludentes
PET	Positron emission tomography
PICK-1	Protein interacting with c-kinase-1
PMSF	Phenylmethylsulfonyl fluoride
PSD	Post-synaptic density
PTN	Post-thalamic nuclei
PVDF	Polyvinylidene difluoride
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SG	Striatum granulosum
SNARE	SNAP receptors
SP	Striatum pyramidale
SR	Striatum radiatum
SM	Striatum moleculare

TBI	Traumatic brain injury
Th	Thalamus
TTC	2,3,5-triphenyltetrazolium chloride

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"The first step to knowledge is admitting that you know nothing"

CHAPTER 1

REVIEW OF LITERATURE

1. Introduction

1.1.Introduction to traumatic brain injury (TBI)

1.1.1. Major causes and consequences of TBI in humans

Traumatic brain injury is one of the leading causes of death and disability among children and young adults in industrialized countries (Waxweiler et al., 1995). In the United States, about 2 million cases are reported every year with approximately 500,000 people being hospitalized (Weight, 1998). In Canada, approximately 50,000 individuals sustain the injury each year (Statistics Canada, 1994). Major causes of injury are principally from motor vehicle accidents, falls, violence or sports related injuries. According to the National Institutes of Health (NIH), the highest incidence is among persons 15 to 24 years of age and 75 years and older, with an additional less striking peak in incidence in children ages 5 and younger (Annonymous, NIH Consensus Statement, 1998). Males are twice more involved in TBI than females. Nevertheless, no one is safe from it, and TBI is a disorder of major public health significance as it affects person of all ages and often results in serious disabilities.

The neurological consequences of TBI are complex, as sensory, motor, and autonomic functions can be compromised. Most of these complications are apparent within the first days or months following injury. Some long-term sequelae include a variety of movement disorders, seizures, headaches, ambient visual deficits, and sleep disorders. Cognitive consequences of TBI are similarly complex, producing different functional problems. Some of the most persistent problems include memory impairment and difficulties in attention and concentration. Deficits in language use and visual perception are common, but often unrecognized. Frontal lobe functions, such as the executive skills of problem-solving, abstract reasoning, insight, judgment, planning, information processing, and organization, are vulnerable to TBI (NIH Consensus Statement, 1998).

1.1.2. Primary injury

TBI is a multifaceted disorder. Primarily, patients with brain injury suffer from surface contusion and laceration, focal or diffuse intracranial hemorrhages, and diffuse axonal injuries. These primary injuries (especially a rise in intracranial pressure, ICP) can evolve into more serious secondary events such as an increase in post-traumatic ischemic burden, oxidative damage, and alterations of endogenous neurochemical mechanisms that may be preventable with proper treatment and the advent of therapeutic strategies.

Table 1.1. *The Glasgow Coma Score. Patients are scored upon their ability to respond to the visual, verbal and physical stimuli and graded accordingly. A total of 13 or higher = mild brain injury; 9 to 12 = moderate injury; 8 or less = severe brain injury. Adapted from Clausen and Bullock (2001) with modifications.*

Glasgow Coma Score (GCS)		
Eye opening	**Verbal response**	**Motor response**
Respond spontaneously ; 4	Confused ; 5	Obeys commands ; 6
Respond to speech ; 3	disoriented ; 4	Localize pain ; 5
Respond to pain ; 2	Inappropriate words ; 3	Withdraws to pain ; 4
No response ; 1	Incomprehensible sounds ; 2	Abnormal flexion to pain ; 3
	No response ; 1	Extension to pain ; 2
		No response ; 1

Immediately after the injury, patients frequently suffer from a combination of hypotension and hypoxemia between the time of injury to being hospitalized, and low cerebral blood flow is also not uncommon. Therefore, it is crucial to maintain sufficient blood flow as well as adequate tissue oxygenation as early as possible (Clausen and Bullock, 2001). Acute assessment of neurological deficits via Glasgow Coma Score (GCS) provides practical early diagnosis of severity of injury (GCSs are indicated in table 1). Following hospitalization, stabilization of all vital systems, establishment of a comprehensive monitoring and a complete assessment of the extent of the primary damage are crucial (Clausen and Bullock, 2001). These are often done with X-ray CT (computerized tomography, see figure 1). Factors that may increase likeliness of complications (secondary injury) via elevating ICP such as epidural, subdural or intracerebral hematomas, or mass lesions are surgically removed at this time. Also, monitoring of ICP and cerebral perfusion pressure (CPP) is necessary and should be followed through.

1.1.3. Secondary injury; post-traumatic ischemic insults and its consequences

The secondary events that follow primary injury are complex, involving a myriad of pathological developments, many of which occur simultaneously. Most times in the patient's brain, tissue oxygenation is compromised. Cerebral metabolic rate for oxygen ($CMRO_2$) can be different from the cerebral metabolic rate for glucose (CMR_{glu}), and hence hypoxic injury can follow trauma even if adequate cerebral blood flow is maintained, especially when acute pulmonary arrest (apnea) is not uncommon in TBI. With reduction of PaO_2 to 65 mm Hg, humans have an impaired ability to perform complex tasks, and a gradual decrease of PaO_2 eventually causes short-term memory loss and loss of unconsciousness (Zauner et al., 2002). Adequate ventilation is therefore essential in reducing the hypoxic damage following trauma.

Hemorrhages resulting from the injury, in addition to increasing ICP by occupying space in the brain, can play a crucial role in post-traumatic ischemic insults, as this often leads to focal regions of vessel spasm (Mendelow et al., 1984), mediated in part by increases in oxidative stress. Such vasospasm can substantially lower

regional blood flow, increasing vulnerability towards the incidence of post-traumatic ischemic events. Although global physiological parameters such as cerebral blood flow (CBF) show no evidence of a direct connection with ischemia in trauma patients, regional vulnerability towards ischemia increases following TBI. In fact, following head injury, increased ischemic brain volume characterized by reduced local CBF and increased oxygen extraction fraction (OEF) can be detected in regions of brain following trauma by voxel-based analysis using positron emission tomography (PET) (Figure 1.1).

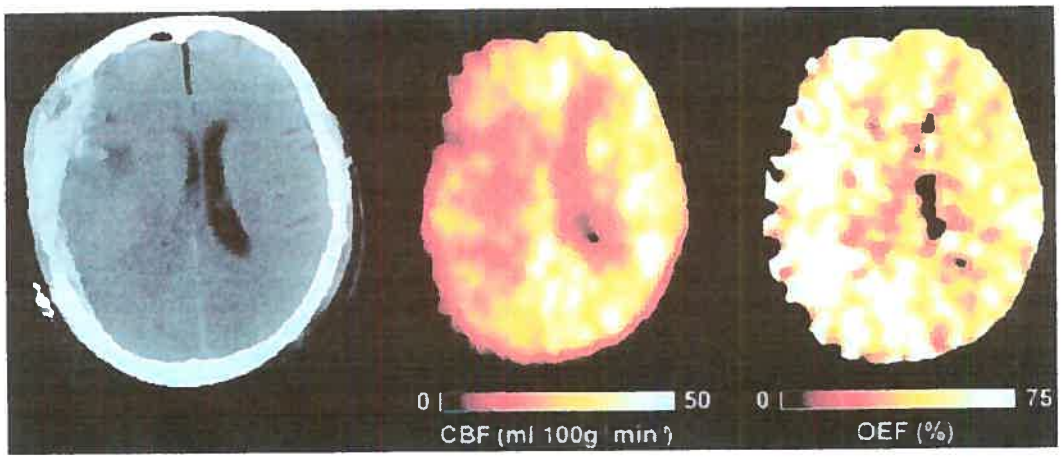

Figure 1.1. *PET images after early head injury. X-ray CT (left), PET CBF (center), and OEF (right) images obtained from a 42-year-old woman 16 hours after injury following evacuation of a subdural hematoma (shown on the left hemisphere). The intracranial pressure was 18 mm Hg, cerebral perfusion pressure was 78 mm Hg, and PaCO2 was 36 mm Hg. Note the small amount of residual subdural blood with minimal midline shift and the marked reductions in CBF and increases in OEF in the cerebral hemisphere underlying the evacuated subdural hematoma. PET, positron emission tomography; CT, computed tomography; CBF, cerebral blood flow; OEF, oxygen extraction fraction. Adapted from Coles et al. (2004).*

The likelihood of ischemic events is further encouraged partly due to the fact that local CBF is unable to increase in response to the heightened metabolic demand. In animal models of TBI, a decrease in local CBF associated with an increase in the local CMR_{glc} has been demonstrated acutely by autoradiography and PET imaging (Ginsberg et al., 1997; Richards et al., 2001). These results point to an early uncoupling of glucose metabolism and blood flow in the contused brain as a potential cause of the increased post-traumatic ischemic burden. Resulting post-traumatic ischemic brain burden could, if unattended, lead to complications that include elevated ICP, reduced CPP, global ischemic brain damage (Mendelow and Crawford, 1997) and, ultimately, brain death.

It has been suggested that CMR_{glu} and $CMRO_2$ reflect astrocytic and neuronal metabolism, respectively, following brain injury (Magestretti et al., 1999). Shortly following cerebral injury, brain lactate levels increase. In normal brain where oxidative metabolism is favored, lactate production from glucose by astrocytes can enter neurons and be converted back to pyruvate, which enters the Kreb's cycle (lactate shuttle hypothesis). Under compromised oxygen levels, oxidative metabolism is impaired, and lactate cannot be metabolized by neurons thus resulting in a buildup of tissue lactate content.

This rise in lactate appears to result from increased anaerobic conversion of glucose to lactate due to the heightened energy demand which may partly represent an attempt to restore normal ionic balance, as application of the Na^+/K^+-ATPase inhibitor oubain is known to attenuate the lactate increase (Kawamata et al., 1995). Ion transport requires a large portion of the energy requirement of the brain tissue, as it has been suggested that approximately 75 % of the increase in oxygen consumption is used to restore adenosine triphosphate levels after neuronal depolarization (Siesjö, 1978). Ionic perturbations mediated by excitatory amino acid-coupled ion channels strongly stimulate energy-dependent ion pumps and thereby activate glycolysis following concussive brain injury (Katayama et al., 1990; Kawamata et al., 1992). Such an increase in glycolysis may be largely responsible for the increased local CMR_{glc} observed following the injury, contributing to a damaging cycle of increased metabolism, oxidative stress, juxtaposed ischemic vulnerability and further ischemic

release of glutamate, potentially leading to a secondary wave of ioninc imbalance and an exacerbation of post-trauma cerebral edema (Figure 1.2).

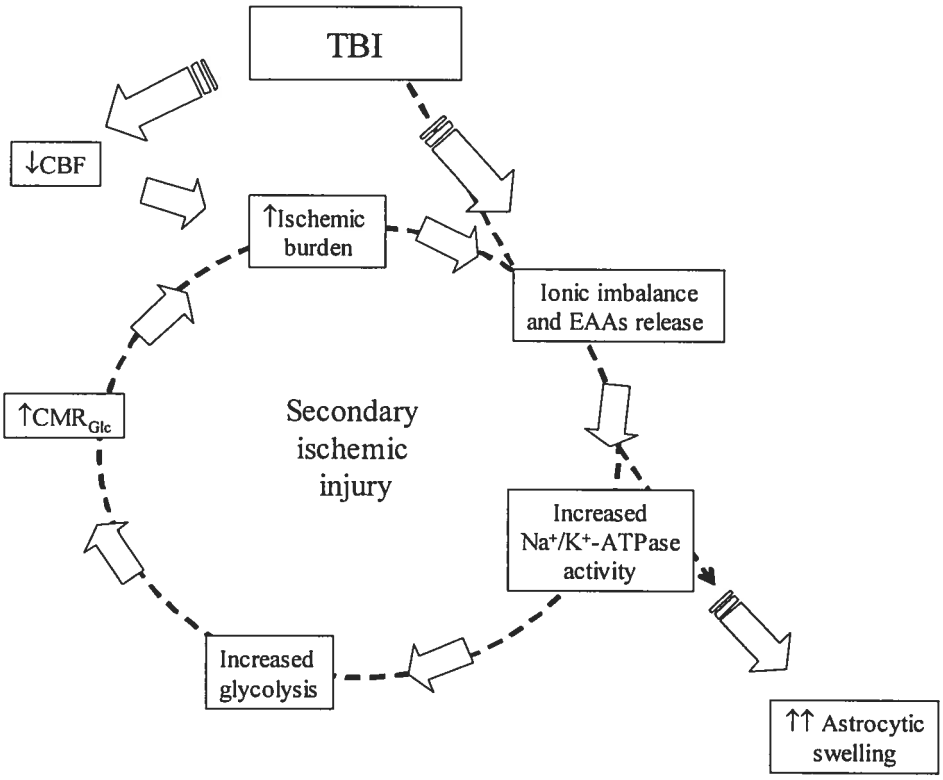

Figure 1.2. *Schematic diagram representing how trauma results in increased ischemic burden and the devastating secondary ischemic injury cycle that could also exacerbate edema. CBF, cerebral blood flow; CMR_{Glu}, cerebral metabolic rate for glucose; EAA, excitatory amino acid. Adapted from Yi and Hazell (2006).*

1.1.4. Animal models of TBI

Given the necessity for understanding the pathophysiology of TBI and screening for new therapeutic interventions, a well-characterized animal model is essential. Successful models of trauma should be able to precisely vary the severity of

injury and the response must be quantifiable and reproducible between different investigators and laboratories (Teasdale et al., 1999; Graham et al., 2000) and replicate the type of severity and injury observed in man.

Due to the variety of human brain injuries and their causes, no single animal model can successfully represent various aspects of human TBI. As a consequence, there are now many different animal models of TBI, each modeling some aspect of human injuries. Pathophysiological and biochemical parameters such as brain edema, increased ICP, elevated brain neurochemicals and hemorrhages can be commonly measured in most animal models of TBI. However, depending on the characteristics and severity of each model, additional parameters may be present or absent. For example, a focal penetrating trauma may consistently show evidence of intracerebral hemorrhage, but this may not be the case in closed head injury. Also, a diffuse closed head model will better replicate pathological characteristics of diffuse axonal injury than a mild penetrating focal model of TBI. In this section, these different models of trauma will be discussed.

1.1.4.1. Inertial acceleration brain injury

A major feature of human closed head injuries is the movement of the brain within the skull rather than a direct impact to the head which can be represented as a rapid rotation of the head. The shearing mechanism created by controlled, fixed angular rotational movement creates acceleration/deceleration forces in the brain that results in diffuse axonal injury located in white matter and brain stem. A non-impact head acceleration model using non-human primates and miniature swine most closely replicates the complex pathobiology of human TBI (Smith et al., 1997; Gennarelli et al., 1982), owing to the similarity in brain mass in these species and hence better resembling physical force applied to human brain upon injury. However, there are certain drawbacks of this model such as the high cost and size of the animal used for experimental purposes. The latter is especially the case since the use of rodents in such a model has been limited due to the fact that the rotational accelerational forces necessary to induce damage to CNS structures increase exponentially with decreasing

brain size, and no injury device exists that can reliably produce a comparable TBI in rodents at present (Marmaru et al., 1994).

1.1.4.2. Indirect brain injury

An explosion causing peripheral blast trauma without direct head injury can also result in significant brain damage with consequent cognitive and motor system deficits. This type of indirect dynamic brain injury can be represented using a rodent model whereby metal cylindrical tubing closed at one end is used to house the anesthetized animal and in which plastic explosive or compressed air generates blast pressure waves near the closed end of the tube (Cernak et al., 2001). The resulting blast wave from detonation of the explosive can cause neuronal damage and swelling in the cerebral cortex as well as astroglial changes in addition to peripheral trauma, and may represent many features of brain damage observed in victims of an explosion (Cernak et al., 2001).

1.1.4.3. Closed head, non-penetrating injury

Impact to the brain can often cause non-penetrating closed head injuries, especially in automobile- and sports-related accidents. The weight drop model, in which the animal (usually rat or mouse) is exposed to a free-falling, guided weight, as well as the impact acceleration model where a steel protection plate is placed over the head of animal to avoid skull fracture from weight drop of high severity, are two such concussion-type animal models, representing diffuse clinical TBI (Cernak, 2005). These models produce concussion-type injuries where focal and diffuse axonal injury is the major pathological outcome. Disadvantages of such models where impact is driven by gravitational force include risks of rebound injury occurring due to a rebounding impactor, and variability of outcome resulting from such an uncontrolled additional insult.

Cold lesion injury, also known as cryogenic (freezing) injury, is caused by exposing the skull and placing a cotton swab soaked in liquid nitrogen or a metal

probe cooled with powdered dry ice upon it. Upon lesioning, the animal exhibits a depression of local cerebral glucose utilization associated with increased brain metabolites such as ATP, phosphocreatine, glucose and lactate in the injured cortex (Pappius, 1981; Buczek et al., 1991) that correlates well with behavioral deficits (Colle et al., 1986). Cold lesion injury in rodents also results in disruption of the blood-brain barrier and reproducible vasogenic brain edema, presumably caused by the rupture of capillary endothelial cells by ice crystals (Murakami et al., 1999).

1.1.4.4. Penetrating head injury

Penetrating head injury with direct brain deformation can be represented by models where the injury is delivered through craniotomy or a direct skull perforation produced by a missile-type force. Direct cortical deformation models include injury caused by a vacuum pulse of clinically relevant duration (<100ms), microinjection of a fluid containing Zymosan (an inert particle macrophage activator), lipopolysaccharide, latex microspheres to induce progressive cavitation, as well as stab wound (Cernak, 2005). Among these, the stab wound (as well as needle puncture) injury is performed with craniotomy followed by a controlled lowering of a needle or a blade. The resulting injury causes limited lesions with reactive gliosis. However, these direct brain-deformation models do not usually cause significant long-term deficits that would be important for representing clinical TBI, because of the absence of extensive diffuse injuries (Cernak, 2005).

The ballistic (gunshot) injury model represents another interesting penetrating head injury model where metal spheres or inflatable projectiles of known weight (bullets) are shot at a precise velocity and distance from the head of the targeted animal. The use of a controlled gunshot enables calculation of energy transferred by the penetrating bullet, facilitating correlation of the resulting neuropathology with injury severity. The gunshot wound model performed on the rat results in volumetric increases in intracranial hemorrhage and lesion size related to injury severity, astrogliosis, macrophage infiltration, neurological deficits, as well as occurrence of cortical spreading depression and seizures (Williams et al., 2005). However, an

increase in missile energy, i.e. high energy wounds also elevates the incidence of respiratory arrest in the cat missile wound model (Carey et al., 1989).

1.1.4.5. Penetrating injury with combined characteristics

The penetrating injury models most frequently used are controlled cortical impact injury (CCII) and fluid-percussion injury (FPI) models. In addition to the fact that these models are the most well-characterized, they have advantages over other penetrating injury models because of the presence of diffuse injury. CCII model is sometimes described as a rigid percussion model. The CCII model utilizes a metal impactor in which the velocity of impact and depth of deformation can be tightly controlled, representing certain advantages over fluid-percussion. The resulting injury from cortical impact is a more focal type of injury compared to that of FPI, which may have implications with respect to behavioral suppression and/or functional alterations resembling coma (Dunn-Meynell and Levin, 1997). The ease of controlling deformation parameters with pneumatically driven devices (time, velocity, and depth of impact) and thus the ability to control the severity of injury is one of the advantages that CCII represents over FPI. In addition, CCII causes significant brain edema following injury that is both vasogenic and cytogenic in nature, as opposed to highly vasogenic brain edema following cold-lesion injury (Unterberg et al., 1997), thus representing the human cortical trauma more appropriately than cold lesion injury. However, one shortcoming of CCII is the lack of brain stem deformation, resulting in minimal mortality as the severity increases, compared to FPI or closed head acceleration/deceleration models.

As is true for CCII, FPI can also be differentiated between a vertex model and that of a lateral model depending on the location of trephination. However, in FPI, the conventional vertex model has a disadvantage compared to the lateral injury model due to the high incidence of mortality as the severity increases, presumably by direct compression of the brainstem. In addition, midline FPI (Dixon et al., 1989) does not reproduce prolonged unconsciousness in animals, a key feature of severe trauma patients. Lateral FPI was originally designed to reproduce coup-contrecoup-type

contusion in animals (McIntosh et al., 1989). Although this original goal was never actually achieved for the most part, lateral FPI is the most popular and well characterized experimental model of TBI today, producing both focal and diffuse injury characteristics (Povlishock, 1994; Hicks et al., 1996) that closely represent closed head injury in humans (McIntosh et al., 1989).

1.1.4.6. In vitro models of TBI

In vitro models using cell cultures offer certain advantages over animal models such as the ease of use, cost efficiency and reproducibility of outcome. In addition, the effect of mechanical stress on a specific cell type can be studied by culturing neurons, astrocytes, and other cells of the central nervous system. There are several types of *in vitro* models of brain trauma that partially replicate the mechanical aspects of transection, compression, acceleration, stretch and shear injury documented in animal models of TBI.

The transection model includes either the use of a plastic stylet to scrape/cut the cells, or use of a laser to perform microsurgery on a single cell. The laser transection model provides exquisite control over injury parameters, but mechanical data (force, strain, strain rate) concerning the process of transection cannot be obtained (Morrison et al 1997).

The compression model attempts to model *in vivo* spinal cord injury. Typically, a weight is dropped on to organotypically cultured spinal tissue. However, this type of model offers little insight into the biomechanics of the tissue injury.

The hydrostatic pressure model utilizes pressure for a given duration to cause damage to cells. Due to the high pressure and long duration used in this model, it may not appropriately represent the actual injury in intact brain. Although there is a transient FPI model of hydrostatic pressure with shorter duration (Shepard et al., 1991), the mechanical injury mechanism associated with *in vi*vo compression trauma may not be properly reproduced by hydrostatic pressure (Morrison et al., 1997).

The acceleration model utilizes a flask of cultured cells exposed to an acceleration force created by an impact pendulum, as high as 200 g (Lucas and Wolf,

1991), aimed at replicating damage following human head injury that may result from shear strain of brain matter due to inertial loading. However, damage to the cells is only detected following repetitive, tangential acceleration thus limiting its use to modeling of injuries associated with multiple impact.

Another model aimed at studying the effects of inertial loading of the brain is the hydrodynamic model. This model shears or stretches cultured cells using hydrodynamic forces generated by a rotating metal plate over the cells. One advantage of the hydrodynamic model is that the cells can be visualized under a microscope during the injury process.

The cell stretch model uses a Flex Plate (Ellis et al., 1995) to cause deformation of wells resulting in a strain to the cells. This model has achieved widespread acceptance and use due to the simplicity of the method.

Easy accessibility, highly reproducible results and relatively low costs as well as tight and facilitated control over the extracellular milieu provide these *in vitro* models of trauma with several advantages over animal models of TBI. In addition, the effect of mechanical stress on individual cell types can be studied *in vitro* by culturing neurons and glial cells separately. However, each *in vitro* model also possesses a certain degree of shortcomings. For example, the shortness of duration that an organotypic culture can be maintained, the fact that primary cell cultures likely represent immature cells because they originate from the brains of embryonic or prenatal animals, and that immortalized cell lines do not appropriately represent normal brain cells. Despite these limiting factors, *in vitro* trauma remains a valid and attractive approach to studying injury processes at the cellular level.

1.1.5. Fluid-percussion injury

The lateral FPI model of TBI in rodents produces injury due to the impact of a rapid fluid bolus which strikes the intact *dura mater*. Minor surgery is performed prior to injury that exposes the *dura* by trephination of a burr hole of 4.8 mm in diameter over the left hemisphere of the brain. FPI can be divided into mild (1-1.5 atmospheric pressure), moderate (2-2.5 atm), or severe (2.5-3.5 atm), measured with a

computerized pressure pulse detecting device, where injury is reproducible and varies according to the severity of insult.

Blood-brain barrier disturbance, loss of consciousness, and altered CBF have consistently been observed in the FPI model. Immediately after trauma, CBF increases in the injured cortex to over 100% baseline (Muir et al., 1992), but then falls to 40-50% of baseline within 15-30 min after injury (Ginsberg et al., 1997), which has been known to last up to 4 h and is associated with an uncoupling of local glucose metabolism. The immediate physiological response to FPI also includes changes in blood pressure (transient hypertension), brief respiratory arrest, elevated ICP, decreased CPP, reduced CBF, and increased vascular resistance (Cernak, 2005). Petechial hemorrhage, axonal damage, subarachnoid hemorrhage, tissue tears followed by focal necrosis (cell loss), vascular damage at grey/white interphase (gliding contusion), apoptosis and necrosis are also reported in FPI (Cernak, 2005). In addition, altered ionic homeostasis and increased intracellular calcium levels, elevated tissue sodium content, impaired potassium equilibrium, EEG depression and motor, behavioural and cognitive impairments are well characterized in FPI.

Although abnormal neurons are detected via Acid Fuscin and Nissl staining methods as early as 10 minutes following insult, visible loss of neurons is not detected until 12 h post injury (Hicks et al., 1996), whereas significant loss of cortical tissue as well as shrinkage of the hippocampal pyramidal cell layer ipsilateral to injury is seen up to one year later (Smith et al., 1997), suggesting the initial loss of neurons takes several hours to develop, and that the lesion is progressive, with a wide window of therapeutic intervention. Use of FPI therefore provides an opportunity to examine detailed mechanisms of secondary injuries leading to neuronal loss.

Reactive astrocytes are detected as early as 1 day following injury that persist up to a month after the insult (Hill et al., 1996). In addition, in regions of atrophy and in the dentate hilus region where bilateral loss of neurons persists, reactive astrocytosis is observed up to 1 year (Smith et al., 1997).

1.2. Role of glutamate, the major excitatory amino acid in the brain

Glutamate is the most abundant excitatory neurotransmitter in the brain. Cerebral glutamate concentration is about 5-15 mmol/kg wet weight, but most of this glutamate is maintained inside the cell, leading to low extracellular glutamate levels (3-4 μM) (Danbolt, 2001). In normal brain, the role of glutamate as an excitatory neurotransmitter is undisputed. However, increased levels of extracellular glutamate following head injury cause over-stimulation of glutamate receptors that may result in series of secondary events, leading to neuronal cell death. Such events can cause prolonged depolarization and subsequent ionic imbalance that could culminate in cytogenic edema, raised ICP, vascular compression and brain herniation, an often fatal complication of severe head injury.

A rise in extracellular glutamate levels may exert its detrimental effect by acting upon glutamate receptors. Cation fluxes due to excessive opening of glutamate receptor channels lead to cellular ionic imbalance and loss of membrane potential. Furthermore, an excessive increase in the intracellular Ca^{2+} via NMDA receptor-operated channels may result in the activation of Ca^{2+}-dependent enzymes as well as increased nitric oxide production. Depending on the energy status of the cell, apoptosis and/or necrosis may be the final outcome. Thus, understanding the structural features of glutamate receptors may provide useful insight into the transduction mechanisms that can lead to excitotoxicity. In the following section, an overview of the action of glutamate on glutamate receptors in the brain will be briefly described.

1.2.1. Ionotropic (NMDA, AMPA/Kainate) receptors

Ionotropic receptors are ligand-gated ion channel proteins of heterotetrameric or pentameric structures each consisting of 3 transmembrane spanning domains and a cytoplasmic-facing re-entrant loop, with an extracellular N-terminal segment and intracellular C-terminal region (Dingledine et al., 1999). Upon activation, ionotropic glutamate receptors induce alterations in the concentration of intracellular ions, and the intracellular carboxy-terminal interacts with a variety of intracellular proteins. There are 3 known types of ionotropic receptors; the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic (AMPA) receptor, the kainate receptor, and the NMDA receptor.

AMPA and kainate receptors are oligomeric ligand-gated ion channels with fast kinetics that are permeable to Na^+ and K^+ and also to low levels of Ca^{2+} (Hollmann et al., 1991). AMPA receptors have a lower glutamate affinity than NMDA receptors, but they have faster kinetics. NMDA receptors contain voltage-dependent divalent and monovalent cation channels more permeable to Ca^{2+} ions. There have been five NMDA receptor subunits identified so far, NR1 and NR2A-D (Hollmann and Heinemann, 1994). NMDA receptors are found both within synapses as well as at extrasynaptic locations. The final localization of NMDA receptors may depend on the anchoring of NMDA receptors to cytoskeletal elements in the synapse via NR1-α-actinin interaction. For NMDA receptors, the presence of NR1 appears invariant, whereas the selection of NR2 A, B, C or D subunits determines the time constants of opening of the channel and modifies the effect of various antagonists.

1.2.2. Metabotropic glutamate receptors (mGluRs)

Metabotropic receptors are subdivided into 3 groups. Group I mGluRs includes mGluR1 and mGluR5, are positively coupled through phosphoinositide-specific phospholipase C to phosphoinositide hydrolysis and intracellular Ca^{2+} mobilization, and sensitive to quisqualate and the cyclic glutamate analogue (1S, 3R)-ACPD. Group II mGluRs includes mGluR2 and mGluR3, are negatively coupled to adenylate cyclase, and also activated by (1S, 3R)-ACPD but less sensitive to quisqualate. Group III mGluRs include mGluR4, mGluR6, mGluR7 and mGluR8 and are also negatively coupled to adenylate cyclase.

1.2.3. Following TBI, extracellular glutamate increases via multiple sources

Following TBI in both experimental and clinical settings, levels of extracellular glutamate increase acutely (Faden et al., 1989; Globus et al., 1995; Palmer et al., 1993; Bullock et al., 1998). Depending on the location of the sampling probe, varying results have been reported in the concentration and the duration of the glutamate increase. In animal models, for example, the increased levels of glutamate

may be short lived, returning to basal values within 4 hrs (Globus et al., 1995). In humans, low levels of sustained increase has been observed (Bullock et al., 1998), thus increasing the window for therapeutic intervention.

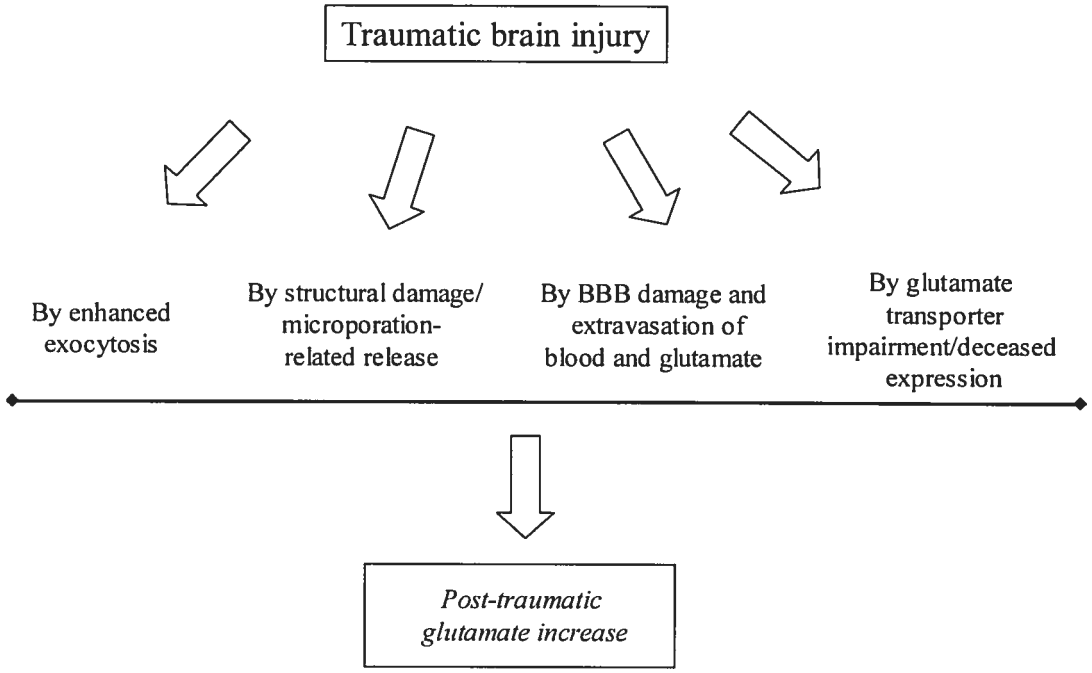

Figure 1.3. *Multiple source of glutamate increase following traumatic brain injury. Post traumatic glutamate levels may rise due to different and complex mechanisms including enhanced exocytosis- or non-exocytosis-related events and impaired glutamate removal from the extracellular space by transporter protein impairment/downregulation. BBB, blood-brain barrier.*

The interstitial glutamate surge following the cerebral insult probably originates from multiple sources (Figure 1.3). Glutamate may move into brain following disruption of the blood-brain barrier, as blood contains higher glutamate concentrations (millimolar) than the interstitial space. Intraparenchymal hemorrhage is often seen following brain trauma, and autoradiography following intravenous

injection of ^{14}C-labeled glutamate revealed marked extravasation of [^{14}C]-glutamate at the site of cortical impact (Koizumi et al., 1997). Release of glutamate may also reflect non-specific development of micropores in the cell membrane, based on the finding that the release of excitatory amino acids is closely related to the release of structural amino acids (Bullock et al., 1998). It is also possible that excessive synaptic release of glutamate may occur following the injury, leading to glutamate overflow into extrasynaptic regions. Last but not least, a decrease in glutamate uptake activity, due either to functional impairment or decreased expression of the transporter protein can also contribute in a major way to the accumulation of extracellular glutamate. Glutamate release as a consequence of reversal of the transporter protein could also result in a rise in interstitial glutamate levels in the core of the lesion (infarction/traumatic core) where damage is most severe (Figure 1.3).

1.2.4. Altered NMDA receptor function following TBI

NMDA receptor is a heterodimeric assembly, thought to be comprised of two NMDAR1 subunits and two or three NR2 subunits. NR1 is the principal constituent of the NMDA channel, homogeneously distributed in the brain that alone can generate a functional pore in vitro (Moriyoshi et al., 1991), whereas the NR2 is a regulatory component with four subtypes (NR2A-D) differentially distributed in the brain and imparting specific electrophysiological properties to the NMDA channel (Osteen et al., 2004). It seems that in understanding functional alteration of NMDA receptor following brain injury requires consideration of the ratio of NR2 subtypes, especially that of NR2A and NR2B, since cortical neurons with more NR2B than NR2A (low NR2A:NR2B ratio) are more sensitive to glutamate, open for a longer period of time, and conduct larger currents than a population with a high NR2A:NR2B ratio (Osteen et al., 2004).

Previous studies using receptor binding techniques have suggested that the function of NMDA receptor might be altered following TBI (Miller et al., 1990, Sihver et al., 2001). An acute decrease of NMDA binding in these studies may have indicate that receptor sensitivity to glutamate could be impaired. However, these

studies did not use the subtype-specific functional attributes of post-injury NMDARs to differentiate between the NR2 subtypes. Osteen and colleges (2004) have shown that a decrease of both subtypes of receptor occurs following FPI, and the NR2A:NR2B ratio indicates that NMDA receptor becomes temporarily more sensitive to glutamate and would remain open longer once activated. This is combined with ^{45}calcium autoradiography and subtype specific antagonists studies suggested that TBI induces molecular changes within NMDA receptor, contributing to the cells' post-injury vulnerability to glutamatergic stimulation (Osteen et al., 2004).

1.2.5. Current perspective on excitotoxic injury

NMDA receptors, and AMPA/kainate receptors to a certain extent, play a major role in excitotoxicity mediated via increase in interstitial glutamate concentration, moreso than metabotropic glutamate receptors. This is understandable considering the functional nature of ionotropic receptors; ion channels permeable to Na^+, K^+ and Ca^{2+}, which are crucial in maintaining the ionic balance of the cell. Over-stimulation of metabotropic receptors may have modulatory roles via secondary messenger pathways, rather than directly contributing in excitotoxicity. As a consequence, application of NMDA antagonists have proven beneficial in animal models of ischemia/reperfusion injury as well as TBI, and undergone clinical trials in the US and elsewhere.

However, past attempts to overcome excitotoxic injury in stroke and trauma patients by application of glutamate receptor antagonists in clinical trials have suffered a great deal of drawbacks (Ikonomidou and Turski, 2002). Many of these antagonists, at beneficial dosages, have major side effects and are potentially dangerous. At lower doses, synaptic tortuosity can hinder efficient drug delivery, making the half-life of these compounds a significant factor. In addition, drugs often suffer from a relatively short therapeutic window for application, and with the fact that many patients can arrive at the hospital for treatment several hours after the insult, their beneficial use can be very limited.

It is thus crucial at present to carefully re-examine excitotoxic mechanisms, particularly those involving glutamate transporters. Since these proteins perform the major role of clearance of glutamate from the extracellular space, it is important to understand their nature in order to develop future therapeutic strategies that may positively impact the consequences of TBI. In addition, our current knowledge of neurotransmitter release process following such an insult to the brain is limited and requires further investigation.

1.3. Role of glutamate transporters in the brain

In brain, glutamate cannot be metabolized within the extracellular space. Instead, glutamate is removed rapidly from the extracellular space by a class of sodium-dependent glutamate transporters known as Excitatory Amino Acid Transporters (EAATs). The maintenance of functional transporters near the synapse is crucial for sequestration of synaptic glutamate, thereby providing a crisp signalling system, as well as preventing potential toxicity by this excitatory amino acid. In the following section, the current literature on sodium-dependent EAATs and their function relative to localization will be considered.

1.3.1. Sodium-dependent EAATs in the brain

1.3.1.1. Different subtypes of sodium-dependent glutamate transporters and their localization

To date, 5 subtypes of sodium-dependent glutamate transporters have been cloned and localized in brain and peripheral tissues: GLAST (Glutamate-Aspartate transporter, [EAAT1], Stork et al., 1992), GLT-1 (Glutamate Transporter-1, [EAAT2], Pines et al., 1992), EAAC-1 (Excitatory Amino Acid Carrier-1, [EAAT3], Kanai and Hediger, 1992), EAAT4 (Fairman et al., 1995) and EAAT5 (Arriza et al., 1997). In the brain, highest levels of GLT-1 protein are detected in the hippocampus and cerebral cortex, whereas the cerebellum expresses highest levels of GLAST (Lehre et al., 1995)

and EAAT4 (Dehnes et al., 1998; Furuta et al., 1997). EAAC-1 concentration is highest in the hippocampal formation, whereas EAAT5 levels are highest in the retina. GLAST and GLT-1 are predominantly localized in astrocytes (Danbolt et al., 1992; Rothstein et al., 1994; Lehre et al., 1995; Reye et al., 2002), while EAAC-1, EAAT4, and EAAT5 appear to be mostly neuronal (Berger and Hediger, 1998; Kugler and Schmitt, 1999; Dehnes et al., 1998; Arriza et al., 1997).

Of the glial transporters, it is estimated that GLT-1 accounts for more than 90% of the total reconstitutable glutamate transport activity in forebrain, with several studies indicating this to be the case. For example, uptake activity in cell homogenates of GLT-1 knockout mice show less than 5 % glutamate transport activity compared to that of wild type (Tanaka et al., 1997). In addition, antibodies to GLT-1 have been shown to immunoprecipitate more than 95% of the reconstitutable transport activity in crude detergent-treated extracts of forebrain tissue (Danbolt et al., 1992; Haugeto et al., 1996). Furthermore, electron microscopy studies using immunogold staining, and other knockout studies involving different glutamate transporter-deficient mice together suggest that the localization of GLT-1 and GLAST account for clearance of most of synaptic glutamate in rat forebrain (Chaudhry et al., 1995; Tanaka et al., 1997; Peghini et al., 1997). On the other hand, the reconstitution process potentially favors conservation of GLT-1 activity, and may inactivate other transporters such as GLAST (Danbolt, 2001); thus our current knowledge on the role of GLT-1 in the brain glutamate clearance may in fact be over-estimated.

Nevertheless, it is less arguable that GLT-1 has the primary glutamate transport role in the forebrain, and accounts for the majority of perisynaptic glutamate uptake. For example, GLT-1 knockout mice develop epilepsy and are vulnerable to cold lesion injury (Tanaka et al., 1997). GLAST is colocalized in the same astrocyte membrane along with GLT-1, and appears to account for an estimated 20% of total hippocampal glutamate transport, when quantification of immunocontent is considered (Lehre and Danbolt 1998). Knockout mice lacking GLAST show loss of motor coordination and an increased edema index following cold lesion injury in the cerebellum (Watase et al., 1999), suggesting an important role in glutamate uptake in this brain region. The neuronal transporter EAAC-1 appears to be expressed in lower

quantities relative to GLT-1 and GLAST, but is expressed at high levels in peripheral organs such as intestine and kidney (Kanai and Hediger, 1992; reviewed by Danbolt, 2001) partly explaining the finding that EAAC-1 knockout mice develop dicarboxylic aminoaciduria and behavioural abnormalities, but display no neurodegeneration (Peghini et al., 1997). This suggests that the neuronal EAAC-1 transporter may play less of a crucial role in removing extrasynaptic glutamate in the brain. EAAT4 appears to be the predominant glutamate transporter in the Purkinje cells of cerebellum (Danbolt, 2001), and is primarily expressed in the soma and dendrites of Purkinje cells (Dehnes et al., 1998; Yamada et al., 1996; Nagao et al., 1997), although low levels of expression have been detected elsewhere (Hu et al., 2003; Ward et al., 2003). EAAT5 has been localized in the retina at high levels by northern blot (Arriza et al., 1997), but details of its protein expression studies are lacking at the present time.

1.3.1.2. Ionic dependence of glutamate uptake

As indicated above, glutamate transporters are Na^+-dependent, with the transmembrane gradients of Na^+ and K^+ providing the major driving force for their function. The currently suggested stoichiometry for the transporters EAAC-1, GLAST, and GLT-1 involves one molecule of glutamate coupled to the cotransport of three Na^+ and one H^+, and the counter transport of one K^+ (Zerangue and Kavanaugh, 1996; Klockner et al., 1993; Levy et al., 1998), with the uptake process generating a net positive charge moving into the cell (Figure 1.4), which can be readily measured using electrophysiological techniques. The binding of glutamate requires extracellular sodium, whereas the internal potassium is necessary for the actual substrate transition. Thus, the binding of glutamate is dependent on the presence of external sodium, and, in the absence of internal potassium, the transport might facilitate exchange between the pools of glutamate into and out of the cell. It has been suggested that this electrogenic property of glutamate transport requires approximately 2% of the total energy consumption of the cerebral cortex (Attwell and Laughlin, 2001).

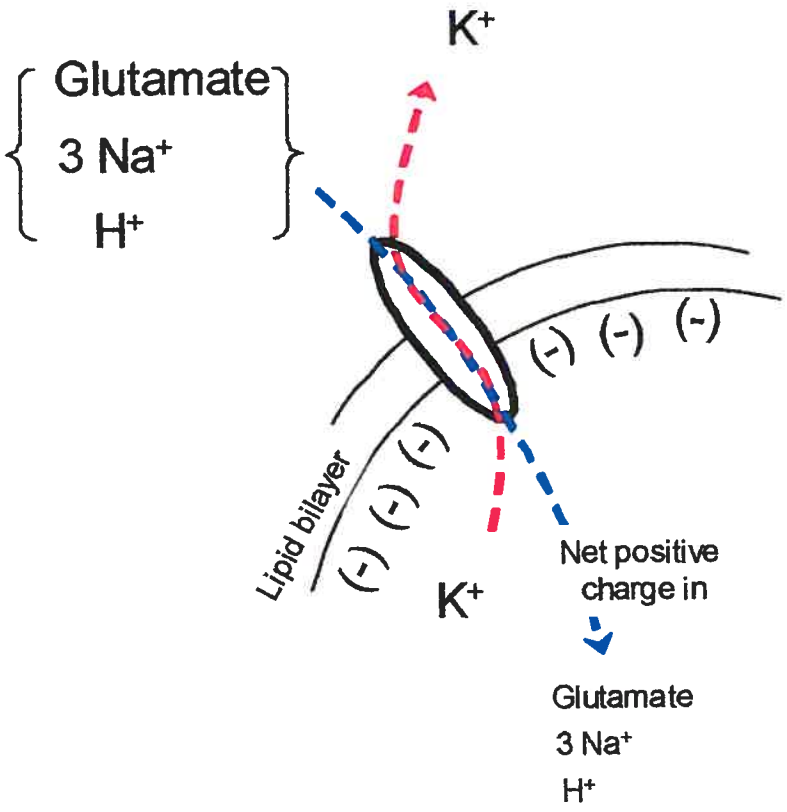

Figure 1.4. *A simplified diagram of the glutamate transporter showing ionic dependence of glutamate transport.*

These transporters also display an anion channel property thermodynamically uncoupled to the substrate transport. The binding of glutamate to the transporters triggers this anionic conductance (reviewed in Danbolt, 2001). The presence of anions inside the transporter ion channels slows down glutamate transport by altering the rate constants for transition into and out of the conducting state (Auger and Attwell, 2000), and may also serve to hyperpolarize the post-synaptic membrane and diminish synaptic activity (Amara and Fontana, 2002). Some members of glutamate transporters such as GLT-1, GLAST and EAAC-1 display low levels of anionic conductance. For others, such as EAAT4 and EAAT5, this anionic conductance is so strong that it accounts for most of the current generated by glutamate uptake process (Fairman et al., 1995; Arriza et al., 1997).

1.3.2. Novel C-terminal splice-variants of GLT-1α

Recently, a number of studies have focused on a new splice variant form of GLT-1, GLT-1v (Chen et al., 2002; Schmitt et al., 2002). This novel form of the predominant splice variant of GLT-1 (GLT-1α) shows high sequence homology with GLT-1α, and was not studied separately from GLT-1α in the past due to a lack of knowledge about this transporter. In addition, GLT-1c, another C-terminal variant of GLT-1 has been partially cloned (Rauen et al., 2004). The putative amino acid sequences of different N-terminal and C-terminal splice variants of GLT-1 are depicted in Figure 1.5.

The existence of novel splice variants with high sequence homology suggests the distinct possibility that, of the >90% reconstitutable glutamate transport activity attributed to GLT-1 in the forebrain, GLT-1c and GLT-1v could contribute significantly. Indeed, since the identity of a nerve terminal glutamate transporter remains elusive (Danbolt, 2001), there is the possibility that one of the splice variants of GLT-1 may prove to be such a nerve terminal glutamate transporter. However, due to lack of detailed information on GLT-1c, only GLT-1v will be discussed in the following sections.

1.3.2.1. GLT-1v

In brain, the sequence homology between GLT-1α and GLT-1v is high, differing only at its C-terminal region (Figure 1.5, Utsunomiya-Tate et al., 1997; Schmitt et al., 2002). Although the differing C-terminal sequence of GLT-1v may indicate alternate transporter properties, expression studies of mouse GLT-1 in *Xenopus* oocytes and rat GLT-1 in HEK (human embryonic kidney) cells showed a considerable high similarity in the functional properties between GLT-1v and GLT-1α (Utsunomiya-Tate et al., 1997; Sullivan et al., 2004). *Xenopus* oocytes expressing GLT-1α and GLT-1v showed no noticeable difference in K_m and V_{max}, but when both splice variants were expressed together, V_{max} showed an increase without changing K_m

(Utsunomiya-Tate et al., 1997), suggesting the two splice variants interact, when expressed in the same cell, altering their transport properties. In addition, it is possible that these two splice variants non-covalently interact and form heteromultimers *in vivo* as suggested by Haugeto et al (1996). Expression of both transporters in HEK293 cells and examination of steady-state currents showed no difference in the apparent affinity of the transporters for glutamate, with GLT-1v being expressed in the membranes of astrocyte somata (Sullivan et al., 2004). Morphological comparison using antibodies directed against GLT-1v suggests that GLT-1v is predominantly located in astrocytes (Reye et al., 2002). Also, double labeling studies have demonstrated that GLT-1α is localized in glial processes, some of which are interposed between multiple types of synapse, whereas GLT-1v is expressed by astrocytic processes at sites not interposed between synapses (Sullivan et al., 2004). Based on their findings, Sullivan and colleagues (2004) have suggested that GLT-1v may not be involved in shaping the kinetics of synaptic signaling in the brain but may be critical in preventing spillover of glutamate between adjacent synapses, thereby regulating intersynaptic glutamatergic and GABAergic transmission.

Interestingly, neuronal expression has been reported for this transporter. For example, Chen et al (2002) showed that GLT-1v protein is expressed at similar levels to GLT-1α in various parts of brain, and in neurons. However, in their subsequent publication, the authors showed messenger RNA expression of GLT-1v in astrocytes whereas GLT-1α showed neuronal expression in the hippocampus (Chen et al., 2004). Later, Berger and colleagues (2005) showed that a subset of neurons in layer VI of cerebral cortex expresses GLT-1v messenger RNA, and the CA3 subfield of hippocampus expresses GLT-1α using *in situ* hybridization. It remains arguable whether the proteins of these transporter variants are expressed in these neurons at the present time.

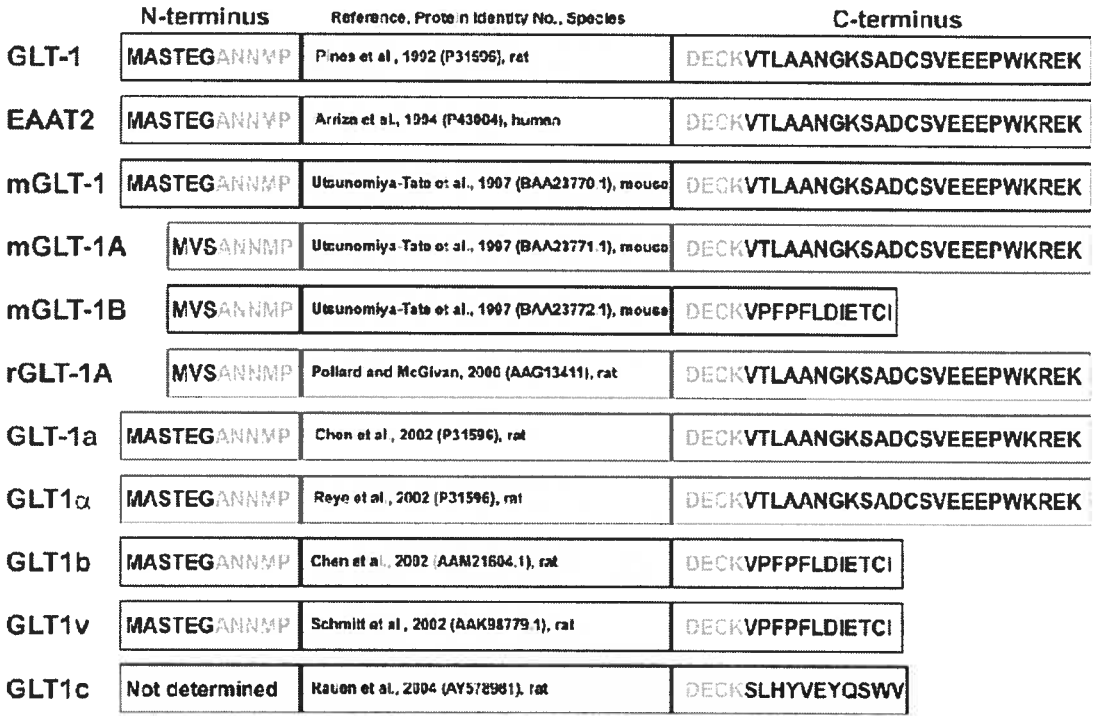

Figure 1.5. *Amino acid sequences and related references of amino- and carboxyl terminal splice variants of GLT-1. Adapted from Rauen et al., 2004.*

1.3.2.2. Functional significance of C-terminal variation

Both GLT-1 transporter activities may result in transduction of intracellular signal MAP kinase activation via N-terminal interacting proteins such as Ajuba (Marie et al., 2002). Many proteins found at synaptic terminals contain sequences of PDZ motifs {(PSD-95/SAP90, discs large (Dig-A, a Drosophila *melanogaster* protein found at the separate junction), *and Zona occludens* (ZO-1, a vertebrate protein found at epithelial cell junctions)} at the C-terminal end of their sequences, a motif known to function in interaction of other post synaptic density proteins. With its differing C-terminal segment containing PDZ sequences, GLT-1v may also interact with the post-synaptic density (PSD) element via *c-kinase-1-interacting protein PICK-1* (Chen and

Rosenberg, 2002). With the possible existence of a novel synaptic neuronal transporter (Danbolt, 2001), as well as the fact that GLT-1v interacts with PICK-1, it is tempting to consider the possibility of this novel splice variant being GLT-1v. At present, the protein expression of GLT-1v with light microscopy shows a pattern of astrocytic staining (Reye et al., 2002; Sullivan et al., 2004) as does mRNA expression with the exception of few subsets of neurons in layer VI of cerebral cortex and CA3 subfield of hippocampus (Chen et al., 2004; Berger et al., 2005). Furthermore, the localization of GLT-1v may be further away from the synapse (Sullivan et al., 2004), suggesting there may be another as yet unidentified synaptic transporter. The PDZ sequences at the C-terminal region of GLT-1v suggests the possibility of different regulatory control of this splice variant form of GLT-1α, or perhaps a different subcellular localization to GLT-1α (Sullivan et al., 2004).

1.3.3. EAAT4, a glutamate transporter with chloride channel-like properties

EAAT4 displays higher affinity for L-glutamate (K_m = 3.3 ± 0.4 μM) with a thermodynamically uncoupled chloride conductance, initially cloned in human cerebellum (Fairman et al., 1995) and later showed to be localized in soma and dendrites of Purkinje neurons in the molecular layer of the cerebellum (Dehnes et al., 1998; Yamada et al., 1996; Nagao et al., 1997). EAAT4 is probably the predominantly expressed neuronal glutamate transporter in the Purkinje cells of cerebellum, with an average density of about 1800 transporter molecules per μm^2 of spine membrane (Dehnes et al., 1998). Unlike other glutamate transporters expressed in the cerebellum, EAAT4 is unevenly expressed in different parasagittal zones with a pattern of expression resembling that of zebrin (aldolase C, Dehnes et al., 1998), but the significance of this distinct pattern of expression is not well understood. In the cerebral cortex, neuronal expression of EAAT4 has also been reported (Massie et al., 2001).

There now exists several lines of evidence for an astrocytic expression of EAAT4 in the brain. For example, Furuta et al (1997) mentioned some astrocytic labeling in the hippocampus, whereas Hu et al (2003) demonstrated the presence of

EAAT4 mRNA and protein in cultured astrocytes and mouse spinal cord and, to a lesser extent, in the forebrain region. In the retina, some immunohistochemical reports suggest EAAT4 is present in the astrocytes (Ward et al., 2004; Fyk-Kolodjief et al., 2004), while in others no such astrocytic labeling was observed (Pignataro et al., 2005). Clearly further investigation is required to properly address this matter.

1.3.4. Glutamate transporters in brain trauma and neurologic disease

A few studies have consistently shown that glutamate transporters may play a role in excitotoxicity that develops following TBI. In traumatic spinal cord injury, glutamate uptake activity in synaptosomal preparations was reduced following the injury, along with 4-hydroxynonenal accumulation that appeared to contribute to inhibition of glutamate uptake (Springer et al., 1997). Transient downregulation of GLT-1 and GLAST levels were reported in the ipsilateral cerebral cortex following controlled cortical impact, with a concomitant reduction in [^{3}H]-D-aspartate binding activity (Rao et al., 1998). Other studies have demonstrated a similar downregulation of GLT-1 and GLAST levels in the ipsilateral and contralateral cerebral cortex, with a rise in CSF glutamate levels, reaching a maximum at 48 h following the injury (van Landeghem et al., 2001). In addition, GLT-1 antisense infusion exacerbated neuronal loss examined at 7 days following TBI (Rao et al., 2001c). These results suggest impairment of transport uptake could follow injury due to downregulation of glutamate transporters that may be partly responsible for the neuronal loss via secondary excitotoxic mechanisms. In addition, accumulation of Na^+ and decreased levels of K^+ in post-traumatic tissue (Soares et al., 1992) further indicate a loss in the Na^+/K^+ gradient and hence diminished glutamate transport capacity, a possible early consequence of TBI.

Following an ischemic insult to the brain, protein levels of glutamate transporters change in a manner that may contribute to delayed neuronal cell death. One study that has provided evidence on the relative role of glutamate transporters in cerebral ischemia was published by Rao et al (2001d). In this report, infusion of the rat with GLT-1 antisense oligonucleotides prior to the generation of transient cerebral

ischemia exacerbated the injury, supporting the notion that maintaining the physiological level of transporter is crucial in minimizing the infarct size. Glutamate transporters have also been reported to reverse direction in the complete absence of energy (Rossi et al, 2000). This condition may be confined to the infarct core where perfusion is sub-ischemic level. In the peri-infarct area, however, downregulation of glutamate transporters level may contribute to additional impairment of glutamate transport. On the other hand, EAAC1 appeared not to contribute to the outcome of injury since antisense infusion did not significantly exacerbate the ischemic infarction (Rao et al., 2001d).

Following 2h of focal ischemia, [^{3}H]-D-aspartate binding in the ischemic core decreased maximally by 20%, reflecting decreased levels of GLT-1 (Gomi et al., 2000). In another study, the transient focal ischemia in spontaneously hypertensive rats lead to decreased GLT-1 and EAAC1 protein and messenger RNA levels, but not GLAST (Rao et al., 2001b).

Following transient forebrain ischemia in rats, quantitative immunoblotting using an antibody against GLT-1 showed a modest 20% decrease in GLT-1 protein content in the hippocampus 6 hours after reperfusion (Torp et al., 1995). *In situ* hybridization revealed decreased GLT-1 messenger RNA signal mainly in the CA1 region of hippocampus that became more pronounced at 24 hr (Torp et al., 1995), suggesting that the down-regulation of glutamate transporters may be one of several factors that contribute to the high sensitivity of CA1 pyramidal cells to post-ischemic damage. In another study, Bruhn et al. (2000) showed conflicting results where GLT-1 messenger RNA in CA1 and CA3 were decreased but the protein levels were unchanged in CA1 and upregulated in CA3 when investigated using quantitative immunohistochemistry. Gottlieb et al (2000) also showed increased EAAC1 levels in the CA1 region with insignificant changes in GLAST and GLT-1 levels by combined techniques of immunoblotting and immunohistochemistry following transient cerebral ischemia in Wistar rats. On the other hand, Rao and colleagues (2000) reported a transient decrease in hippocampal GLT-1 with progressive loss of hippocampal EAAC1 levels and a transient loss of cerebral cortex EAAC-1 level following transient forebrain ischemia in the gerbil. These variations in results are likely due to

differences in the duration and severity of ischemia i.e. the time of artery occlusion along with the species differences which together contribute to the heterogeneity of results. In summary, therefore, cerebral ischemia may result in down-regulation of one or more glutamate transporters, thus increasing the vulnerability of surrounding neurons to excitotoxicity (Danbolt, 2001).

In amyotrophic lateral sclerosis (ALS), alterations in glutamatergic neurotransmission were initially reported (Rothstein et al., 1990, 1992) that were subsequently linked to a loss of EAAT2 in the brain and spinal cord of these patients (Rothstein et al., 1995), a feature of the resulting motor neuron degeneration. The splice variant GLT-1v is interestingly upregulated in ALS, whereas the opposite is observed in Huntington's disease (Maragakis et al., 2004). Recent studies have demonstrated that overexpression of EAAT2 in mice containing an additional Cu^{2+}/Zn^{2+} superoxide dismutase (SOD1) mutation known to result in this ALS-like disorder produced a delay in onset of the condition (Guo et al., 2003). However, these animals did eventually develop the disorder, suggesting that loss of EAAT2 is a likely contributor to, but not the cause of, neuronal damage. Loss of glutamate transport, EAAT2, and splice variants of this transporter have also been described in Alzheimer's disease (Masliah et al., 1996; Li et al., 1997), in experimental Huntington's disease (Behrens et al., 2002), and in a model of the Guamanian disorder ALS-parkinsonism dementia complex (Wilson et al., 2003).

1.4. Introduction to complexins: expression, localization and mechanism of action

As mentioned earlier, the glutamate rise following an insult to the brain could originate from different sources (Figure 1.3). In a glutamatergic synapse, for example, increased presynaptic release owing to the rise in one or several constituents of exocytotic machinery could lead to increased extrasynaptic glutamate. In the following section, presynaptic proteins termed complexins will be introduced, along with the explanation of its importance on molecular machinery of exocytosis and its potential role in neurodegenerative disease.

1.4.1. Role of complexins in the molecular mechanism of neurotransmitter release

Complexins are 15-16 kDa cytosolic proteins. In the cerebral cortex and hippocampus, electron microscopic studies have shown that complexin I (also known as synaphin II) is found in axosomatic terminals and complexin II (also known as synaphin I) in axodendritic terminals (Ishizuka et al., 1999). This localization of each complexin to presynaptic neurons of inhibitory and excitatory synapses has permitted differential assessment of excitatory and inhibitory pathways (Harrison and Eastwood, 1998; Eastwood et al., 2001). Both complexins show highly conserved sequence homology between species, with 100% homology between rat, mice and human complexin II (McMahon et al., 1995). Complexins are thought to compete with the chaperone protein α-SNAP (soluble *N*-ethylmaleimide-sensitive factor-attachment protein) for binding to SNAP receptors (SNAREs) at the presynaptic terminal (McMahon et al., 1995). These SNAREs consist of the synaptic vesicle protein synaptobrevin as well as the synaptic membrane proteins SNAP-25 and syntaxin 1 (Figure 1.6). Further studies have indicated that complexins rapidly bind to the SNARE complex in an anti-parallel configuration and with high affinity (Pabst et al., 2002; Chen et al., 2002), stabilizing the fully assembled SNARE complex. This key step enables the rapid Ca^{2+}-evoked neurotransmitter release (Chen et al., 2002), promoting interaction of transmembrane region between *v*-SNARE (synaptobrevin/VAMP) and *t*-SNARE (SNAP-25) and thereby facilitating neuronal exocytosis (Hu et al., 2002). However, the exact contribution of complexins to neurotransmitter release has been a controversial issue, with some studies indicating that complexins play a negative role in exocytosis, and others suggesting a positive role in neurotransmitter release (Ono et al., 1998; Tokumaru et al., 2001; Reim et al., 2001).

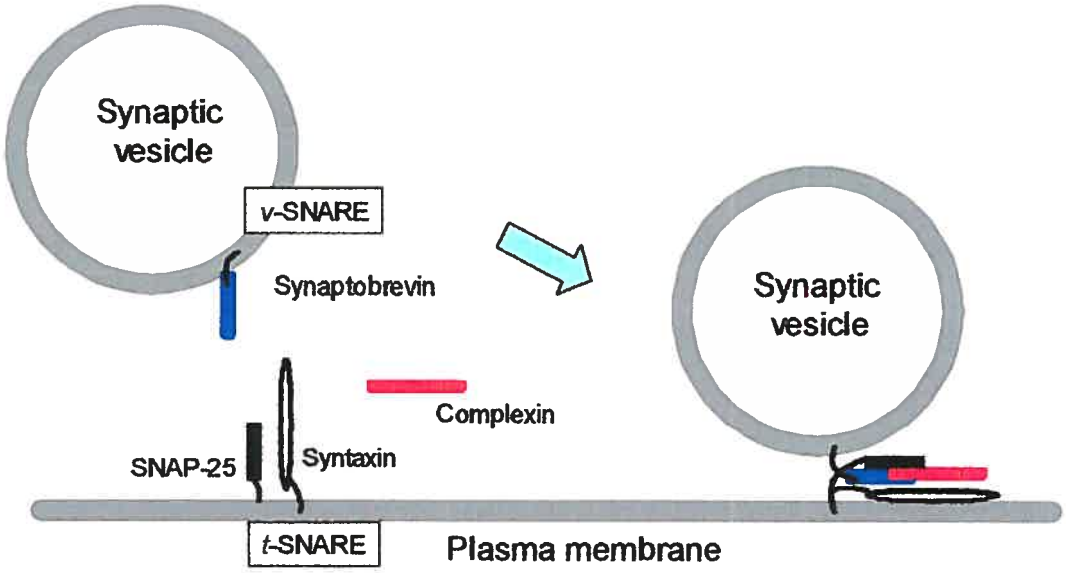

Figure 1.6. *Simplified diagram depicting synaptic vesicle in proximity to the plasma membrane.*

Complexins are thought to play a negative role in exocytosis by competing with α-SNAP (McMahon et al., 1995), thereby interfering with dissociation of the SNARE complex and subsequent recycling of vesicles required for further exocytosis. In addition, the finding that injection of an anti-complexin II antibody into presynaptic neurons of the *Aplysia* buccal ganglion stimulates neurotransmitter release, whereas recombinant complexin II had the opposite effect, suggests a potential inhibitory role in the exocytosis machinery (Ono et al., 1998). Furthermore, over-expression of complexins in PC12 cells resulted in reduced exocytosis of secretory vesicles (Itakura et al., 1999). However, the dissociation of SNARE complex occurs regardless of whether complexin is bound or not (Pabst et al., 2000), and a small oligopeptide specifically disrupting the complexin binding to the SNARE complex showed a positive role in the molecular machinery of exocytosis (Tokumaru et al., 2001), supporting an opposite positive role for this complexin. Indeed, the finding that Ca^{2+}-dependent synchronous release of neurotransmitter is significantly impaired in double

knockout mice lacking both complexins (Reim et al., 2001) further supports the notion that complexins are required for synaptic vesicle release.

1.4.2. Role of complexins in neurodegenerative disease

In psychiatric disease such as major depression, bipolar disease and schizophrenia (Sawada et al., 2002; Eastwood and Harrison, 2001), complexin levels have been measured in post-mortem brain tissue in order to assess the extent of excitatory or inhibitory involvement in the region of interest. Moreover, in a transgenic mouse model of Huntington's disease, progressive loss of complexin II was detected that later colocalized in a subpopulation of neuronal intranuclear inclusions with ubiquitin (Morton and Edwardson, 2001). In addition, expression of mutant Huntingtin, the gene responsible for Huntington's disease, blocked exocytosis in PC12 cells via depleting complexin II levels, which could be rescued by overexpressing complexin II in the same cells (Edwardson et al., 2003), indicating the potential importance of this novel presynaptic protein in neurodegenerative disease states. Thus far, studies have not yet been published on the potential involvement of complexins in brain injury such as stroke or TBI, making such an investigation of the role of complexins in excitotoxicity of particular interest.

1.5. General mechanisms of oxidative injury after TBI

Oxidative stress has been defined as a disturbance in the pro-oxidant/antioxidant balance in favor of the former, leading to potential damage (Sies, 1991). Natural formation of oxidants during mitochondrial electron transport, auto-oxidation of some neurotransmitters such as norepinephrine or dopamine, and initiation of events during trauma, hypoxia or ischemia, can result in oxidant formation and subsequent tissue damage (Warner et al., 2004).

Oxidative stress can be traced primarily to formation of superoxide and nitric oxide. Superoxides are mostly generated from electron leak during mitochondrial electron transport, perturbed mitochondrial metabolism and inflammatory responses to

injury (Halliwell and Gutteridge, 1999), whereas nitric oxides formation is both constitutive and inducible. Brain injury-induced nitric oxide overproduction is in part caused by glutamatergic-mediated increases in intracellular calcium concentration, resulting in a calmodulin-dependent upregulation of nitric oxide synthetase (Garthwaite et al., 1988).

Nitric oxides can elicit nitrosative damage (Espey et al., 2000) via independent nitrosylation of protein heme sites or through its reaction products with oxygen or other nitrogen oxides (Warner et al., 2004). The major oxidative stress produced by excessive nitric oxides is its reaction with superoxide to result in peroxynitrite formation (Beckman et al., 1990) and its involvement in the Fenton reaction (Warner et al., 2004). An example of peroxinitrite-associated damage of protein function would be its reported action on inhibition of glutamate transporter (Trotti et al., 1996). Superoxides can also cause oxidative damage of iron/sulfur clusters of aconitase (Gardner and Fridovich, 1991), an important enzyme in the tricarboxylic acid cycle. Hydroxyl radical, peroxinitrite and peroxinitrite-derived products all have the potential to react with and damage most cellular targets including lipids, proteins and DNA. As such, reactive oxygen species/nitrosative species have been most commonly tracked by measuring stable metabolites such as nitrates/nitrites or footprints of the reactions of these molecules with lipids (thiobarbituric acid adducts, 4-hydroxinonenal), DNA (8-hydroxyguanine), or proteins (nitrotyrosine).

1.5.1. Role of antioxidants in TBI

The brain has potent defenses against superoxides such as endogenous tripeptide glutathione and enzymatic antioxidants superoxide dismutases or Catalases. These antioxidants regulate superoxide concentration by dismutation of superoxide to hydrogen peroxide (superoxide dismutase) which is then converted to water (peroxidases such as glutathione peroxidase and peroxiredoxin) or dismuted to water and oxygen (Catalase, see Warner et al., 2004). Although these antioxidants are reported to be up regulated following brain injury, endogenous antioxidant capacity can be overwhelmed, leading to increased superoxide and hydrogen peroxide

concentration (Warner et al., 2004), and exogenous application of antioxidants following experimental TBI have shown its beneficial effect in minimizing the injury volume and neuronal loss post trauuma.

Ates and colleges (2006) have tested the effect of antioxidant Resveratrol on traumatized rats using weight drop model. They showed that the trauma caused a significant increase in Malondialdehyde (MDA), Xathine oxidase (XO), nitric oxide levels and decrease in glutathione level as compared to control group. Resveratrol administration significantly reduced MDA, XO and nitric oxide levels, increased GSH level, and also attenuated tissue lesion area. In another study, pre-treatment with LY341122 (a potent inhibitor of lipid peroxidation and an antioxidant) attenuated histopathological damage, as indicated by smaller contusion volumes and a reduction in damaged cortical neurons after fluid percussion brain injury in rats (Wada et al., 1999). Stilbazulenyl nitrone, a novel antioxidant, has been shown to improve neurological deficit and reduce histopathological damage after TBI (Belayev et al., 2002).

Bromocriptine, a dopamine D_2 receptor agonist with significant antioxidant properties, was tested on cognition, histopathology, and lipid peroxidation in a rodent model of focal brain trauma. Bromocriptine-treated group was significantly more adept at locating a hidden platform in the water maze compared to the vehicle group and also exhibited a greater percentage of surviving CA_3 hippocampal neurons following TBI (Kline et al., 2004). In addition, a well-known antioxidant *N*-acetylcysteine (NAC) was effective in reducing the injury volume after TBI via its antioxidant-capacity. NAC treatment also attenuated the induction of antioxidant protein heme-oxygenase-I in astrocytes following TBI (Yi and Hazell, 2005)

1.5.2. *N*-acetylcysteine and brain injury

NAC is a cysteine analogue antioxidant (glutathione precursor) with multi-therapeutic uses and is commonly used to treat the hepatotoxic consequences of acetaminophen overdose (Kelly, 1998). NAC can protect against reactive oxygen

species-induced injury either by promoting glutathione synthesis (Corcoran et al., 1986) or by direct scavenging action (Aruoma et al., 1989).

NAC treatment (163mg/Kg, i.p.) given within 1 h post-TBI greatly restored brain and brain mitochondrial glutathione levels (Xiong et al., 1999). The treatment also markedly restored mitochondrial electron transfer, energy coupling capacity, calcium uptake activity and reduced calcium content absorbed to brain mitochondrial membranes when examined post-TBI. This study suggests that NAC administered at an early stage post-injury can effectively attenuate TBI-induced mitochondrial dysfunction, and this protective effect of NAC may be related to its restoration of glutathione levels in the brain. Same authors also reported later on that NAC administered after severe CCI reduced appearance of shortened BCl-2 and BAX proteins, suggesting anti-apoptotic effect of NAC (Xiong et al., 2001).

NAC at 163 mg/kg has previously been shown to enhance hippocampal neuronal survival after transient forebrain ischemia in rats (Knuckey et al., 1995), and same dose was reported to restore cerebrovascular resposiveness following traumatic brain injury in cats (Ellis et al., 1991).

In neonatal rats, the combined treatment of NAC and hypothermia attenuated hypoxic-ischemic injury (Jatana et al., 2006). Cuzzocra and colleges (2000) have reported that NAC treatment resulted in reduced edema index and attenuated myelopeoxidase formation as well as reducing NT positive staining resulting in increased survival and reduced hyperactivity following MCAO in Mongolian gerbils.

However, when dogs were subjected to global ischemia induced by cardiac arrest, NAC did not show any neurological score improvement (Silbergleit et al., 1999). In addition, Thomale and colleges have used the same amount of NAC (163mg/Kg, i.p.) given after CCI of moderate severity and showed that there were no significant differences in the ICP, brain edema as well as the size of the contused volume in rats (2006). Using a single clip spinal cord model in rats, NAC showed no MDA content change after 1h (Kaynar et al., 1998). It is likely that some differences may exist between varying models, animals, conditions of the study, as well as the person conducting the study.

1.6. Thesis objectives

Elevated levels of glutamate following brain trauma exert their effect via excitotoxic mechanisms and perturbation in ionic gradients across the cell membrane. This constitutes one of the major secondary injuries following TBI. However, the precise mechanism(s) by which this increase occurs following TBI is still unknown, with different potential routes suggested (Figure 1.3).

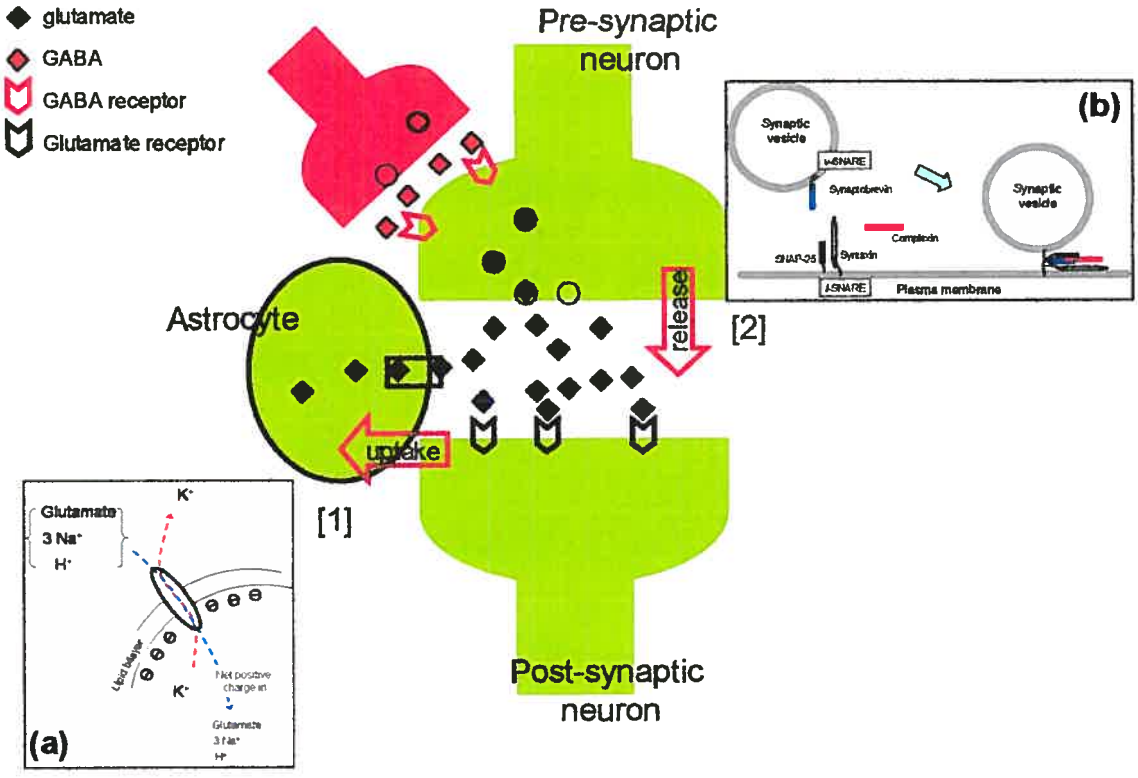

Figure 1.7. *Simplified glutamatergic synapse showing two major ways in which an increased synaptic glutamate concentration can occur: 1) reduced uptake capacity via impaired glutamate uptake (inset a) and, 2) enhanced release of glutamate via elevated levels of complexins (inset b).*

This thesis will investigate the possibility that glutamate transporters and complexins contribute to elevated levels of glutamate following FPI. This will be achieved by formulating and testing two hypotheses: 1) Alterations in glutamate transporter subtypes occur following TBI that may contribute to the increased glutamate and neuronal loss, and 2) TBI produces changes in complexin levels that may contribute to increased release of neurotransmitters and excitotoxic damage (Figure 1.7).

CHAPTER 2

EXCITOTOXIC MECHANISMS AND THE ROLE OF GLUTAMATE TRANSPORTERS IN THE PATHOPHYSIOLOGY OF TRAUMATIC BRAIN INJURY

2.1 Article 1

Early loss of the glutamate transporter splice variant GLT-1v in rat cerebral cortex following lateral fluid-percussion injury.

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ABSTRACT

Glutamate transporter proteins are essential for the control of interstitial glutamate levels, with an impairment of their function or levels being a major potential contributor to excitotoxicity. We have investigated the effects of lateral fluid-percussion on the levels of the glutamate transporter proteins GLT-1α, its splice variant GLT-1v, GLAST, and EAAC1 in the rat in order to evaluate their pathogenetic role in this model of traumatic brain injury (TBI). Immunoblot analysis revealed neuronal loss in the cerebral cortex was accompanied by a 54% decrease in GLT-1v 6 hr following the insult which progressed to an 83% loss of the transporter after 24 hr. No changes in GLT-1α, GLAST or EAAC1 were observed in this brain region at either time point. GLT-1v content was also decreased by 55% and 68% in the hippocampus and thalamus respectively at 6 hr post-injury, but recovered fully after 24 hr in both brain regions. In contrast, levels of GLT-1α were increased in the hippocampus at 6 hr and 24 hr post-TBI. These alterations in transporter protein content were also confirmed using immunohistochemical methods. Our results show for the first time a pattern of early, dynamic changes in the levels of GLT-1 transporter splice variants in different brain regions in this trauma model. In addition, correlation of GLT-1v levels with both neuronal cell loss and α-internexin content in the injured cortex suggests that loss of this novel glutamate transporter may be a key factor in determining cerebral vulnerability following this type of brain injury.

Running title: GLT-1 transporters and TBI

Keywords: traumatic brain injury, glutamate transporter, excitoxicity, GLT-1, fluid-percussion

INTRODUCTION

Glutamate excitotoxicity plays a crucial role in the secondary damage caused by traumatic brain injury (TBI), in which increased glutamate concentrations in the extracellular fluid have been reported (Katayama et al., 1990; Zauner et al., 1996; Koizumi et al., 1997; Matsushita et al., 2000). The neurotoxic effect of extracellular glutamate is well documented (Di et al., 1999; Choi, 1992; Clausen and Bullock, 2001), in which over-excitation by glutamate can lead to subsequent neuronal loss. Our understanding of the exact mechanism by which increased levels of glutamate in the interstitial compartment can cause excitotoxic damage continues to evolve, with recent studies reporting that administration of NMDA receptor antagonists such as memantine, phencyclidine or MK-801 exert a protective effect following TBI (Hayes et al., 1988; McIntosh et al., 1989; Okiyama et al., 1998; Rao et al., 2001b), supporting the notion of a glutamate-mediated process in this disorder.

Clearance of extracellular glutamate is mainly executed by astrocyte glutamate transporters. The concentration of extracellular glutamate necessary for neurotoxicity in cultured neurons increases substantially when astrocytes are added (Nicholls and Attwell, 1990), providing evidence that astrocytic glutamate transporters play an important role in the clearance of this amino acid. In addition, antisense knockdown studies reveal that GLAST and GLT-1, but not EAAC1, produce excitotoxic damage and increased susceptibility to seizures and injury (Rothstein et al., 1996; Tanaka et al., 1997; Rao et al., 2001c,d), with a body of evidence supporting an important role of astrocytic glutamate transporters in a variety of brain disorders that include ischemia, thiamine deficiency, amyotropic lateral sclerosis, acute liver failure, and Alzheimer's disease (Rothstein et al., 1995; Torp et al., 1995; Knecht et al., 1997; Li et al., 1997; Hazell et al., 2001). In brain, five subtypes of glutamate transporter have so far been cloned: GLAST (Stork et al., 1992), GLT-1 (Pines et al., 1992), EAAC1 (Kanai and Hediger, 1992), EAAT4 (Fairmen et al., 1995) and EAAT5 (Arriza et al., 1997). Of these, GLAST and GLT-1 are predominantly localized in astrocytes (Danbolt et al., 1992; Rothstein et al., 1994; Lehre et al., 1995), while EAAC-1, EAAT4, and EAAT5 appear to be mainly neuronal. Previous studies have indicated that GLT-1 and GLAST

levels are decreased following cortical impact injury (Rao et al., 1998; van Landeghem et al., 2001), and that intracortical infusion of glutamate or knockdown of GLT-1 by antisense oligonucleotides exacerbates TBI (Di et al., 1999; Rao et al., 2001c). Reports of the existence of alternative GLT-1 sequences (Utsunomiya-Tate et al., 1997) have been followed by studies involving a splice-variant known either as GLT-1v (Schmitt et al., 2002) or GLT-1B (Chen et al., 2002; Reye et al., 2002). Since this GLT-1B variant form is different to a GLT-1B form described by Utsunomiya-Tate and colleagues, we will use the term GLT-1v in subsequent references to this protein to avoid confusion, and GLT-1α to refer to the original splicing form of GLT-1 (Sullivan et al., 2004). GLT-1v appears to be predominantly localized to astrocytes rather than neurons (Reye et al., 2002) but has also been reported in neurons and microglia (Schmitt et al., 2002; Chen et al., 2002). While the importance of GLT-1 (GLT-1α and GLT-1v) in the clearance of glutamate is well established (Rothstein et al., 1996), where its activity accounts for ~90% of glutamate uptake (Kanai, 1997), the impact of GLT-1v alone on cerebral disorders remains largely unknown.

In the present study, we investigated early effects of TBI in rats following lateral fluid-percussion, a clinically relevant model of TBI that reproduces many of the key pathophysiological features exhibited in head-injured patients (McIntosh et al., 1989). Using specific antibodies directed against GLAST, GLT-1α, EAAC1 and GLT-1v in association with immunoblotting and immunohistochemistry, levels of these transporters were evaluated in relation to neuronal damage in the injured cerebral cortex, hippocampus, and thalamus. Our results suggest an important role for GLT-1v in the sequelae of events following TBI.

MATERIALS AND METHODS

Materials

Protease inhibitor cocktail, mouse monoclonal antiserum to β-actin, rabbit anti-goat IgG secondary antibody, 3,3'-diaminobenzidine (DAB), nickel ammonium sulfate and sodium azide were purchased from Sigma Chemical Co (St. Louis, MO, USA). Polyvinylidene difluoride (PVDF) membranes and broad-range protein markers

were purchased from Bio-Rad Laboratories (Hercules, CA, USA), enhanced chemiluminescence (ECL) kits were purchased from New England Nuclear (Boston, MA, USA) and X-OMAT autoradiography film was purchased from Kodak (Ile des Soeurs, Quebec, Canada). Affinity-purified goat polyclonal antisera to glial fibrillary acidic protein (GFAP), to α-internexin, and to synaptophysin, biotinylated donkey anti-rabbit and donkey anti-goat IgG secondary antibodies were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA). Affinity-purified mouse monoclonal antiserum to glutamine synthetase (GS) was purchased from BD Biosciences Pharmingen (San Diego, CA, USA). HRP-coupled goat anti-rabbit and goat anti-mouse IgGs were purchased from Promega (Madison, WI, USA). HRP-coupled donkey anti-goat IgG was purchased from Promega Corporation (Madison, WI, USA).

Trauma Model

The lateral (parasagittal) model of traumatic brain injury used in this study has previously been described in detail (McIntosh et al., 1989). All procedures were undertaken with the approval of the Animal Ethics Committee of Hôpital Saint-Luc and the University of Montreal, and were conducted in accordance with the guidelines set out by the Canadian Council on Animal Care. Male Sprague-Dawley rats weighing 350-375 g were housed under constant conditions of temperature, humidity and 12:12 h day/night cycle, and fasted overnight. The following day, animals were injected with sodium pentobarbital, (65 mg/kg, i.p.) and placed in a stereotaxic frame. A 4.8 mm diameter craniotomy, centered between bregma and lambda, and 2.5 mm lateral to the superior sagittal sinus was made over the left hemisphere using a trephine drill, and a hollow female Luer-Lok fitting secured with dental cement. Rats were subsequently allowed to recover overnight and, the following day, reanesthetized with 2% isofluorane and exposed to lateral fluid-percussion injury of moderate severity (2.0-2.5 atm). The pressure wave applied was recorded using custom-written software (FP302; AmScien Instruments, Richmond, VA, USA) to monitor reproducibility. All animals recovered from transient apnoea with good peripheral circulation within 45 sec of injury. Sham controls received identical treatment but without exposure to trauma.

Immunoblotting

At the appropriate time, a total of 17 rats (shams, n = 5; 6 hr post-TBI, n = 6; 24 hr post-TBI, n = 6) were sacrificed by decapitation. The brains were removed and rapidly frozen in isopentane at -20°C. Regions of interest (injured cerebral cortex, hippocampus, and thalamus) were dissected out at 0°C over dry ice in a coronal block of tissue extending -3.8 to -5.8 mm from bregma (Figure 1). Tissues were homogenized in ice cold TE buffer (50mM Tris, 150mM NaCl) with protease inhibitor cocktail (Sigma Chemical Co., St. Louis, MO, USA) and phenylmethylsulfonyl fluoride (PMSF) using a Teflon glass homogenizer at 1000 rpm, 25 strokes, on ice. Resulting homogenates were centrifuged for 30 min at 15 000 x g, 4°C, and the supernatants designated "crude cytosolic fraction" were separated into another tube. Pellets were washed with TE buffer, recentrifuged, the supernatant discarded, and solubilized in RIPA buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, pH 8.0 and protease inhibitor cocktail). The latter were designated "crude membrane fraction". Protein content was determined by the method of Lowry et al (1951).

Immunoblotting assays were performed as previously described (Hazell et al., 2001). Briefly, aliquots of the crude membrane fraction were added to equal volumes of sample buffer containing dithiothreitol (200 mM) and proteins were separated using discontinous SDS-polyacrylamide gel electrophoresis (SDS-PAGE) consisting of 10% resolving and 4% stacking gels at a constant 40 V overnight and transferred to PVDF membranes by wet transfer. Membranes were subsequently incubated in a blocking solution consisting of 5% dry non-fat milk in TTBS (10mM Tris, 100mM NaCl, and 0.1% Tween-20) followed by incubations with antisera against GLT-1α (1:10,000), GLT-1v (1:1,000), GLAST (1:5,000), EAAC1 (1:1,000), α-internexin (1:1,000), synaptophysin (1:200) or actin (1:10,000) in blocking solution for 1 hr, washed three times in TTBS, and then reblocked for 45 min. Details of characterization of rabbit polyclonal antisera to the C-terminal domains of GLT-1v, GLAST and GLT-1α have previously been described (GLT-1α and GLT-1v, Reye et al., 2002; GLAST, Pow and Barnett, 1999). The antibody against GLT-1v was raised using the synthetic peptide PFPFLDIETCI corresponding to the carboxyl terminus of GLT-1v in mouse (Genbank

accession number NP035523) (Utsunomiya-Tate et al., 1997) and the same carboxyl terminus region in rat (Chen et al., 2002; Schmitt et al., 2992). A gift of rabbit polyclonal antiserum against EAAC1 (C-491) was kindly provided by Dr. Niels Danbolt. In some cases, incubation with primary antisera was performed at 4°C. After further washes in TTBS, membranes were incubated in HRP-coupled goat anti-rabbit (1:20,000), goat anti-mouse (1:20,000), or donkey anti-goat IgG (1:20,000) secondary antiserum in blocking solution for 1 hr. Membranes were then washed five times with TBS, treated with ECL kit reagents according to the manufacturer's instructions and then exposed to X-OMAT film. Optical density measurements were performed using a microcomputer-based image display system (Imaging Research Inc., St. Catherines, Ontario, Canada). Linearity of the relationship between optical density and protein concentration was verified using appropriate standard curves. Coomassie staining of the acrylamide gels was performed to ensure equal loading of proteins.

Immunohistochemistry and Histology

Rats (all groups, n = 4) were deeply anesthetized with pentobarbital (60 mg/kg) and perfused transcardially as described previously (Hazell et al., 2001). Brains were removed and post-fixed overnight in neutral-buffered formalin containing 4% formaldehyde, 0.5% sodium phosphate buffer, and 1.5% methanol, pH.7.0. Coronal sections (-3.8 to -5.8 mm relative to bregma) of 40-μm thickness were cut using a vibrotome according to the rat brain atlas of Paxinos and Watson (1998). Immunohistochemistry was performed according to Hazell and colleagues (2001). Briefly, sections were incubated for 10 min in phosphate-buffered saline (PBS) containing 0.3% hydrogen peroxide to block endogenous peroxidase activity. Tissue sections were then washed in PBS (3 x 10 min), blocked for 20 min in 0.5% Triton X-100 and 5% donkey serum, and incubated with or without 0.5% Triton X-100, along with 5% donkey serum and primary rabbit antisera directed against GLT-1α (1:1,000), GLT-1v (1:500), GLAST (1:400), or EAAC1 (1:1,000), goat-derived antisera against α-internexin (1:250) or synaptophysin (1:100), or mouse-derived antisera against GFAP (1:1,000) or GS (1:5,000) at 4°C for 24 hr. The following day, sections were washed (3 x 10 min) and incubated for 1 hr with biotinylated donkey anti-rabbit/goat

IgG in PBS containing 0.5% Triton X-100 followed by washing (3 x 10 min), and incubation for 1 hr in streptavidin-HRP conjugate in PBS with 0.5% Triton X-100. Sections were then again washed (3 x 10 min), followed by incubation with DAB (0.05%) in PBS containing 25 mg/ml nickel ammonium sulfate for signal enhancement and in the presence of H_2O_2 (0.03%) for 2-10 minutes. Sections were then mounted on Superfrost Plus slides (Fisher Scientific, Ottawa, Ontario, Canada), cleared in xylene, and coverslipped with permount. Negative controls consisted of omission of primary or secondary antibody, resulting in loss of immunoreactivity.

Vibratome-cut sections were stained with cresyl violet for evaluation by light microscopy. Neuronal cell numbers were assessed by counting and averaging four adjacent grid areas (0.06 mm^2 each) at a magnification of 400X.

RESULTS

Immunoblot studies on glutamate transporters

Levels of the glutamate transporters GLT-1α, GLT-1v, GLAST, and EAAC1 were studied in brain regions with known vulnerability to damage in this model by immunoblotting. In the injured cerebral cortex, a decrease in GLT-1v was observed at 6 hr and 24 hr following TBI which was not apparent in the case of GLT-1α, GLAST, or EAAC1 (Fig. 2A). Quantitative analysis revealed that, relative to actin content, GLT-1v levels were decreased by 54% ($p < 0.01$) compared to shams 6 hr following injury, and further reduced by 83% ($p < 0.01$) after 24 hr (Fig. 2B). Both GLT-1v and GLT-1α blots exhibited bands corresponding to monomer and multimer forms of the transporter proteins. In quantitating the levels of these two transporters, we considered both monomer and multimer forms of the proteins. However, the multimer bands showed no obvious differences between sham and TBI groups (data not shown).

Examination of the hippocampus and thalamus revealed that levels of GLT-1v were transiently reduced by 55% and 68% ($p < 0.01$) respectively 6 hr following TBI (Fig. 3A,B). However, GLT-1v levels returned to normal 24 hr post-injury in both areas. Interestingly, in the hippocampus, the decrease in GLT-1v 6 hr post-TBI was accompanied by an increase in the levels of GLT-1α (40%, $p < 0.05$) that was also

present at 24 hrs following trauma (57%, $p < 0.05$) (Fig 3A,B). However, this effect on GLT-1α was not evident in the thalamus. In contrast, GLAST and EAAC1 levels were found to be unchanged in both the hippocampus and thalamus (data not shown).

Histological Analysis

Histological assessment following cresyl violet staining revealed considerable damage to the affected portion of the cerebral cortex (area directly below the site of trauma, Fig. 1), particularly in the deeper layers, associated with a loss of neurons 6 hr following induction of TBI (Fig. 4A-C). Further loss of neuronal staining and the presence of gliosis were observed after 24 hr in this area. In the hippocampus, damage was most evident in the stratum pyramidale of the CA2 subfield, at 6 hr and 24 hr following trauma (Fig. 5G-I). Neuronal damage was also observed as early as 6 hr following TBI in the stratum granulosum of the dentate gyrus, stratum pyramidale of the CA1 region, and in the posterior lateral and lateral ventral thalamic nuclei. Quantitatively, in the 6 hr post-TBI group, decreases in neuronal cell numbers of 47%, 40%, and 41% compared with shams were observed in the cortex, hippocampus, and thalamus, respectively (Table 1). Twenty four hours following TBI, animals showed further decreases in neuronal cell numbers of 76%, 64%, and 66% in these three brain regions (Table 1).

Immunohistochemistry

Examination of GLT-1v immunoreactivity in the injured cortex revealed a considerable loss of immunoreactivity, particularly in layers V and VI that seemed to coincide with the area where most of the damage was observed (Fig. 4D-F). Immunolabeling appeared to be "patchy" in the other layers. In the hippocampus and thalamus, loss of immunoreactivity was transient (Fig. 4G-F,J-L), with the loss of immunoreactivity occuring mostly in the perikarya. In the hippocampus, GLT-1v immunostaining appeared to be localized to astrocytes (Fig. 5A, inset) with decreased immunoreactivity being evident at 6 hr post-TBI compared to sham controls. Loss of immunoreactivity was particularly obvious in the stratum radiatum at this time (Fig. 5B). On the other hand, GLT-1α immunoreactivity was increased after 6 hr compared

with sham animals and this effect was maintained at 24 hr post-injury (Fig. 5D-F). GLT-1α immunostaining was particularly high in the CA2 subfield where considerable neuronal damage was observed, with intense labeling of astrocytic processes around the pyramidal cells (Fig. 5H,I). Comparison of GLT-1v immunoreactivity with that of the astrocyte-specific GS and the astrocyte-localized cytoskeletal protein GFAP revealed a close similarity in labeling patterns in both the injured cortex and hippocampus (Fig. 6), providing further support of an astrocytic localization for GLT-1v.

Relationship between GLT-1v and neuronal damage in the injured cerebral cortex

In order to further evaluate the extent of neuronal damage we measured α-internexin protein content in the injured cortex by immunoblotting (Fig. 7A). Levels of α-internexin were decreased by 17% in the 24 hr post-TBI group compared to sham levels ($p < 0.05$). In contrast, no changes in synaptophysin levels were detected in either the 6 or 24 hr-post TBI groups compared to control rats (data not shown). Examination of the relationship between GLT-1v transporter levels and neuronal counts showed a significant ($p = 0.0001$) positive correlation during the first 24 hr following brain injury (Fig. 7B). Assessment of the relationship between levels of GLT-1v and α-internexin also showed a significant ($p = 0.0358$) positive correlation during the first 24 hr following TBI (Fig. 7C).

DISCUSSION

In this study we have provided the first evidence of alterations of astrocytic glutamate transporter levels following lateral fluid-percussion injury to the brain. Figure 8 shows a summary of the present findings. Our results indicate a loss of the transporter splice variant GLT-1v, but not GLT-1α, in the injured cerebral cortex, occurring as early as 6 hr post-TBI, and which is sustained for up to 24 hr following the insult. This is particularly interesting as GLT-1 is considered to be functionally the most important glutamate transporter subtype in brain, accounting for at least 90% of the glutamate uptake capacity under normal conditions (Haugeto et al., 1996; Tanaka

et al., 1997), and comprising activity associated with both GLT-1α and the novel splice variant GLT-1v. Given the degree of cortical damage that ultimately results following moderate fluid-percussion injury (Hicks et al., 1996), our findings of an early loss of GLT-1v levels suggest that this transporter is an important pathogenetic contributor in this brain region. At the same time, levels of other glutamate transporters such as GLAST and the neuronal transporter EAAC-1 were unaffected in this brain region, further emphasizing this issue. These findings with GLT-1v are also similar to a recent study in which treatment of mice with a cycad neurotoxin as a model of ALS-PDC led to levels of GLT-1v being more affected than GLT-1α (Wilson et al., 2003).

Previous studies have reported increases in extracellular glutamate concentration in the cerebral cortex following TBI (Nilsson et al., 1990; Kanthan and Shuaib, 1995; Bullock et al., 1998). Although Rao and colleagues (1998) have reported a downregulation of glutamate transporters (GLT-1α and GLAST) following brain trauma (Rao et al., 1998), we have been unable to reproduce these findings in the present study. However, this discrepancy may be due to the fact that the Rao study involved a cortical impact model, which typically causes more damage to the tissue than in our case. Use of lateral fluid-percussion injury provides a more clinically relevant model of brain injury in terms of the mechanical shearing forces developed at the time of injury (McIntosh et al., 1989; Hicks et al., 1996), and thus loss of GLT-1v but not GLT-1α and GLAST may be more representative of the consequences of moderate concussive injury. Our findings of a strong positive correlation between GLT-1v levels and neuronal counts ($r = 0.78$, $p = 0.0001$) suggests this glutamate transporter splice variant may contribute to cell death in the injured region of the cortex. To investigate this further, we used the neurofilament protein α-internexin as a supporting index of neuronal damage. Examination of the relationship between levels of GLT-1v and α-internexin revealed a significant positive correlation in the injured cortex ($p = 0.03$, $r = 0.51$), adding further credence to the concept that loss of this glutamate transporter plays a role in the neuronal damage to this area. Previous studies have established that neuronal cell death is associated with decreased levels of this axonal protein, including loss of α-internexin in the spinal cord of patients with

amyotrophic lateral sclerosis in which motor neurons undergo cell death (Wong et al., 2000), in post-mortem brain (Fountoulakis et al., 2001), and in association with neuroblastoma cell death (Chan et al., 1998). Downregulation of GLT-1v may therefore be a major contributing factor to the rise in interstitial glutamate levels observed following the ensuing neuronal damage and cell loss. On the other hand, it is possible that loss of GLT-1v may be a consequence of the neuronal injury process itself; similar effects have been observed with GLT-1α (Levy et al., 1995). Indeed, the patchy nature of the GLT-1v immunostaining in the injured cortex appears to be consistent with the pattern of cresyl violet staining of the same area, indicating that some astrocytes may either be affected by the neuronal damage or may die in this region. It is also possible that GLT-1v and neuronal cell loss may simply be correlated with no direct cause-effect relationship. Further studies are therefore necessary to investigate in more detail the mechanisms involved in the regulation of this novel glutamate transporter splice variant.

In the hippocampus, GLT-1v was transiently decreased at 6 hr post-TBI but recovered to normal values after 24 hr. On the other hand, GLT-1α levels were upregulated at 6 hr following TBI and maintained at elevated levels up to 24 hr. One interpretation of these findings is that hippocampal astrocytes may be attempting to compensate for the early loss of GLT-1v by upregulation of GLT-1α in order to maintain adequate removal of glutamate from the extracellular space. Interestingly, the thalamus also experienced a similar transient decrease in GLT-1v levels but without the accompanying elevation of GLT-1α. This is despite the fact that the degree of neuronal loss experienced in the hippocampus was very similar to that of the thalamus following TBI. Although the reason for this interregional difference regarding GLT-1α is unknown, differences in normal excitatory/inhibitory influences or differences in the levels of these two splice variants of GLT-1 between these two regions may ultimately determine the astrocyte response. In addition, studies have demonstrated that soluble factors released by neurons (Gegelashvili et al., 1997), possibly as a consequence of damage, can upregulate GLT-1α; thus reversal of the downregulation of hippocampal and thalamic GLT-1v levels at 24 hr may be due to a similar process.

Developing gliosis following neuronal cell death may also contribute to this later recovery of GLT-1v.

While the functional interactions between GLT-1α and GLT-1v are presently unclear, the study by Utsunomiya-Tate and colleagues (1997) using *Xenopus* oocytes demonstrated that the transporter activities of both GLT-1 splice variants were maximal when the two transporters are expressed in equal proportion. In addition, previous studies have reported the existence of both monomer and multimer forms of GLT-1α (Haugeto et al., 1996). Together, these findings suggest that when both transporters are co-expressed in the same cell membrane, they may interact and form multimers, providing a means of quickly modifying transporter activity in which individual cells can adjust the local efficiency of the transport system. In the present study, we have also observed multimer bands for both GLT-1α and GLT-1v. Such findings support this concept of an interaction between both transporters. Further studies will be required to examine the precise nature of the functional relationship between these two variants of GLT-1.

In the present study, GLT-1v immunoreactivity appears to be predominantly localized to astrocytes, consistent with earlier findings (Reye et al., 2002). In addition, GLT-1v downregulation is transient in the hippocampus and the thalamus, making it more likely that the antibody is targeting primarily astrocytes rather than neurons, the latter of which die under these conditions. On the other hand, Schmitt and colleagues (2002) have reported that a similar splice variant of GLT-1 appears to be concentrated in neurons. We have not been able to confirm these findings with GLT-1v in the three brain regions examined in the present study. Nevertheless, such reports along with ours suggest the existence of different forms of GLT-1, perhaps with cell type- and region-specific roles to play in terms of glutamate clearance.

The fact that GLT-1v is expressed in astrocytes near glutamatergic synapses, and that this splice variant might form heteromultimers with GLT-1α to regulate the uptake of glutamate transport activity together suggest an important contribution of this splice variant to the development of excitotoxicity in this model. Our results showed no change in the levels of EAAC1 in any of the 3 regions examined in this study. EAAC1 is reported to be absent in glutamatergic nerve terminals (Danbolt et al.,

1998), and antisense knockdown of EAAC1 did not exacerbate the degree of neuronal damage arising from transient focal cerebral ischemia (Rao et al., 2001a). Such findings lead us to conclude that this transporter does not participate to any great extent in the neuronal damage arising from this type of fluid-percussion injury. Our observations of a loss in GLT-1v suggest an important role for this transporter splice variant in TBI. Additional studies focussing on the controlling mechanisms responsible for this effect should yield further insight into the exact role of GLT-1v, along with potential therapeutic strategies for improved treatment of the resulting neuronal damage.

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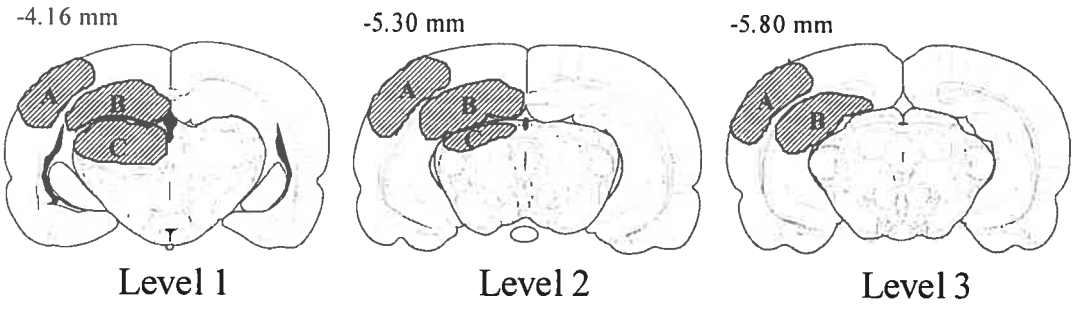
Table 1. Regional neuronal cell counts

	Cerebral cortex	Hippocampus (CA2)	Thalamus (PTN)
Sham (n = 5)	130 ± 4	75 ± 2	80 ± 4
6 hr post-TBI (n = 6)	68 ± 11[a]	45 ± 3[a]	47 ± 5[a]
24 hr post-TBI (n = 6)	30 ± 6[a,b]	27 ± 6[a,b]	27 ± 2[a,b]

Values are mean ± SEM. [a] significantly different compared to sham ($p < 0.001$), [b] significantly different compared to 6 hr post-TBI group ($p < 0.05$); ANOVA with *post-hoc* Bonferroni correction. PTN, posterior thalamic nuclei.

Figure 1

Regions of interest in the rat brain following lateral fluid-percussion injury. Levels 1-3 are representative of coronal sections ranging from -4.16 to -5.8 mm relative to bregma according to the rat brain atlas of Paxinos and Watson (1998). Shaded areas show the underlying injured frontal parietal cortex (A), hippocampus (B) and posterior dorsal lateral thalamus (C) utilized in immunoblotting studies.



Level 1 Level 2 Level 3

Figure 2.

Effect of fluid-percussion injury on the levels of four glutamate transporters in the injured cerebral cortex following TBI. A) Immunoblots show representative bands for GLT-1α, GLT-1v, GLAST, EAAC1 and actin. B) Quantitative densitometric analysis of shams (n = 5), and 6 hr (n = 6) and 24 hr (n = 6) following injury. Levels of GLT-1v are decreased as early as 6 hrs with further down-regulation at 24 hr post-injury. Samples of the injured cortex were dissected out and processed as described in the "Materials and Methods" section. Lanes were loaded with equal amounts of protein in each case (GLT-1α, 10 μg; GLT-1v and EAAC1, 50 μg; GLAST, 35 μg; actin, 20 μg). *$p < 0.05$ compared with sham levels (ANOVA with *post-hoc* Dunn's test).

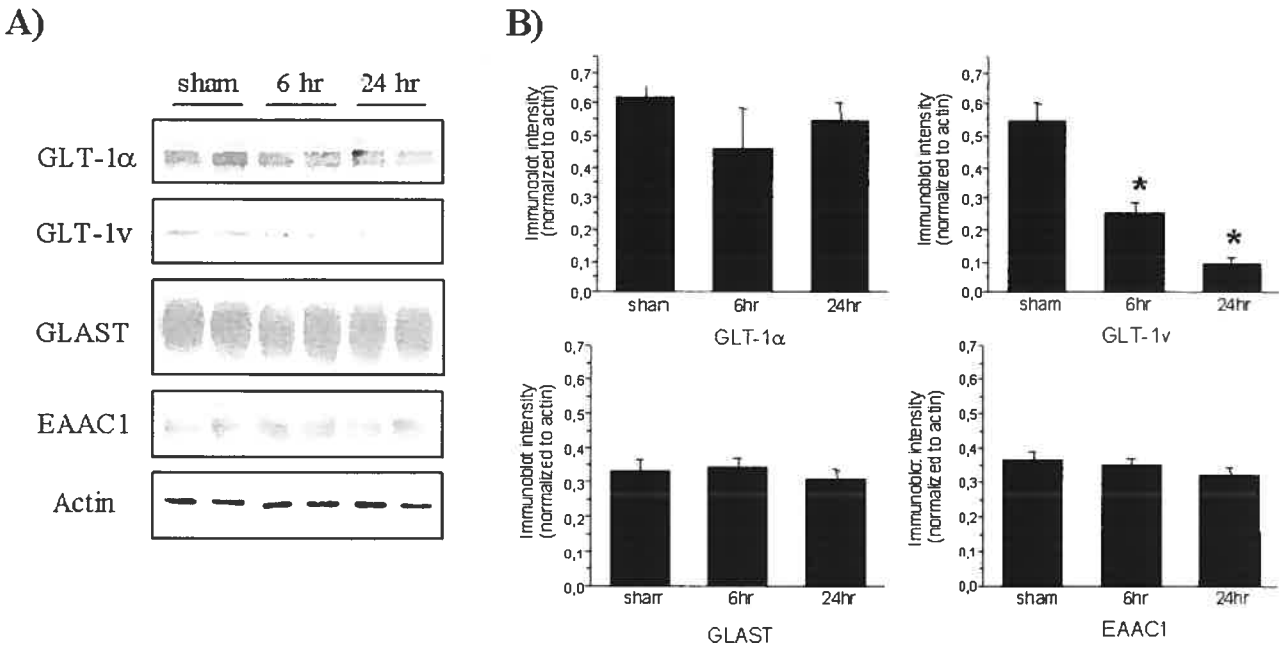
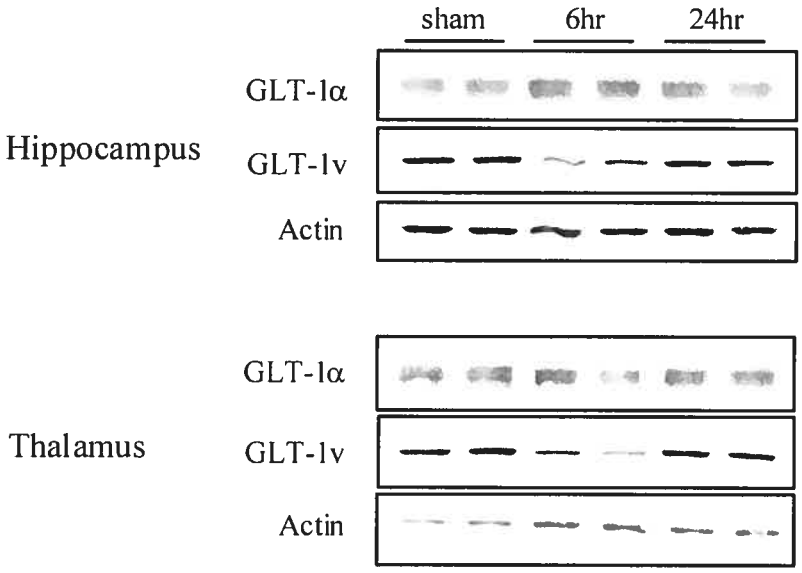

A)
sham
6 hr
24 hr
GLT-1α
GLT-1v
GLAST
EAAC1
Actin
B)
Immunoblot intensity (normalized to actin)
sham
6hr
24hr
GLT-1α
GLT-1v
GLAST
EAAC1
*
*

Figure 3.

Effect of fluid-percussion injury on GLT-1 splice variant levels in the ipsilateral hippocampus and thalamus following TBI. A) Immunoblots show representative bands for GLT-1α, GLT-1v and actin. B) Quantitative densitometric analysis of shams (n = 5), and 6 hr (n = 6) and 24 hr (n = 6) following injury. Levels of GLT-1v were transiently decreased in both brain regions at 6 hr post-injury, whereas GLT-1α showed an up-regulation in hippocampus, but no change in thalamus. *$p < 0.05$ compared with sham levels (ANOVA with *post-hoc* Dunn's test).

A)


sham
6hr
24hr
Hippocampus
GLT-1α
GLT-1v
Actin
Thalamus
GLT-1α
GLT-1v
Actin

B)

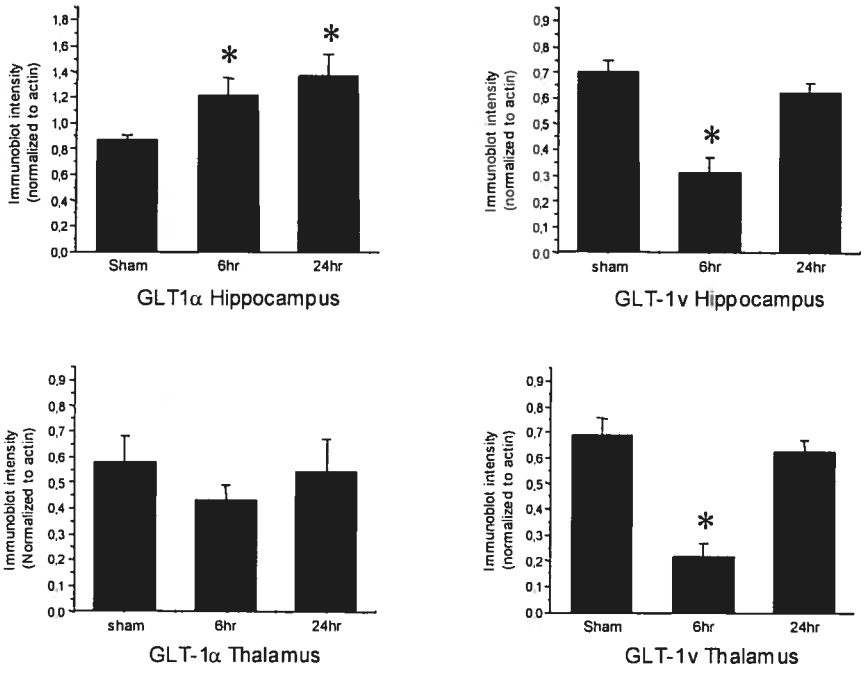

Immunoblot intensity (normalized to actin)
Sham
6hr
24hr
GLT1α Hippocampus
Immunoblot intensity (normalized to actin)
sham
6hr
24hr
GLT-1v Hippocampus
Immunoblot intensity (Normalized to actin)
sham
6hr
24hr
GLT-1α Thalamus
Immunoblot intensity (normalized to actin)
Sham
6hr
24hr
GLT-1v Thalamus

Figure 4.

Regional changes in GLT-1v immunoreactivity following fluid-percussion injury. Photomicrographs of coronal sections from sham (n = 4), 6 hr post-TBI (n = 4), and 24 hr post-TBI animals (n = 4). Cresyl violet staining of the injured cortex (Cx) shows progressive neuronal loss at 6 and 24 hr following trauma compared to sham (A-C). This is accompanied by a decrease in GLT-1v immunoreactivity at 6 hr (E, arrows) with further loss at 24 hr (F, arrows). In the hippocampus (Hc) and thalamus (Th), GLT-1v immunoreactivity is transiently decreased at 6 hr (H, K) compared to sham (G, J) (arrows), but recovers to sham levels at 24 hr (I, L). Bar represents 200 μm.

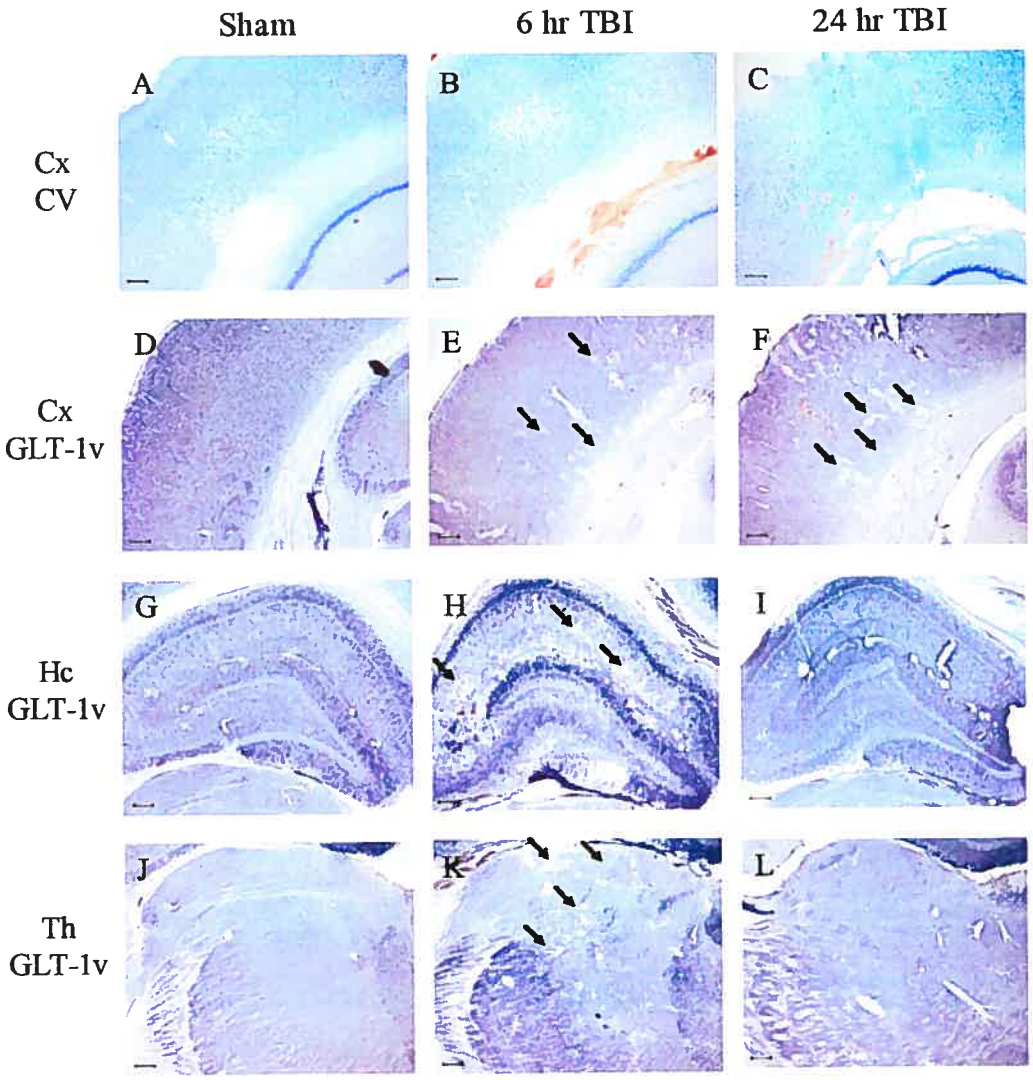

Sham
6 hr TBI
24 hr TBI
A
B
C
Cx
CV
D
E
F
Cx
GLT-1v
G
H
I
Hc
GLT-1v
J
K
L
Th
GLT-1v

Figure 5.

Differential changes in GLT-1α and GLT-1v immunoreactivity in the hippocampus. Photomicrographs show coronal sections from representative sham (n = 4), 6 hr post-TBI (n = 4), and 24 hr post-TBI animals (n = 4). GLT-1v is transiently lost at 6 hr, returning to sham levels at 24 hrs (A-C), whereas GLT-1α immunoreactivity is increased in the ipsilateral hippocampus (D-F). Note staining of astrocyte processes for GLT-1v (A) and considerable loss of GLT-1v immunoreactivity in stratum radiatum (SR) at 6 hr post-trauma (B). Considerable neuronal damage is apparent in the CA2 hippocampal subfield, with more intense GLT-1α immunoreactivity in astrocytic processes around pyramidal cells compared to sham (G-I). SG, stratum granulosum; SP, stratum pyramidale. Bars represent 80 μm in A-F, and 40 μm in G-I.

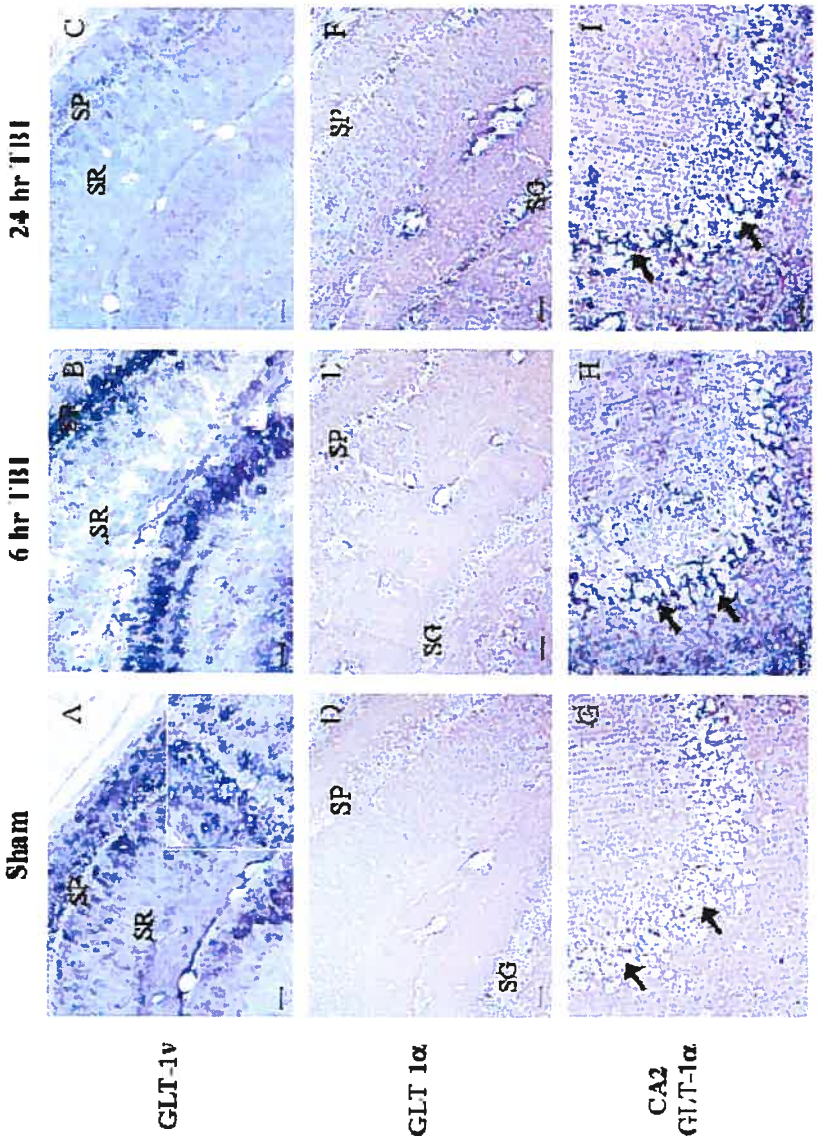

Sham
6 hr TBI
24 hr TBI
A
B
C
D
E
F
G
H
I
SP
SR
SG
GLT-1v
GLT 1α
CA2
GLT-1α

Figure 6.

Pattern of GLT-1v, glutamine synthetase (GS), and glial fibrillary acidic protein (GFAP) immunoreactivities in the cerebral cortex and hippocampus of a representative sham animal (n = 4). Comparison of the immunostaining profiles for GLT-1v (A-D) and the two astrocyte-specific proteins GFAP (E-H) and GS (I-L) show a close similarity in which the processes of astrocytes are well defined, with an absence of neuronal staining in cerebral cortex, and stratum pyramidale (SP) and stratum moleculare (SM) of hippocampus. D, H, and L are from the SM layer of panels C, G, and K respectively. Bar represents 100 μm in A, C, E, G. I, and K, and 25 μm in B, D, F, H, J, and L.

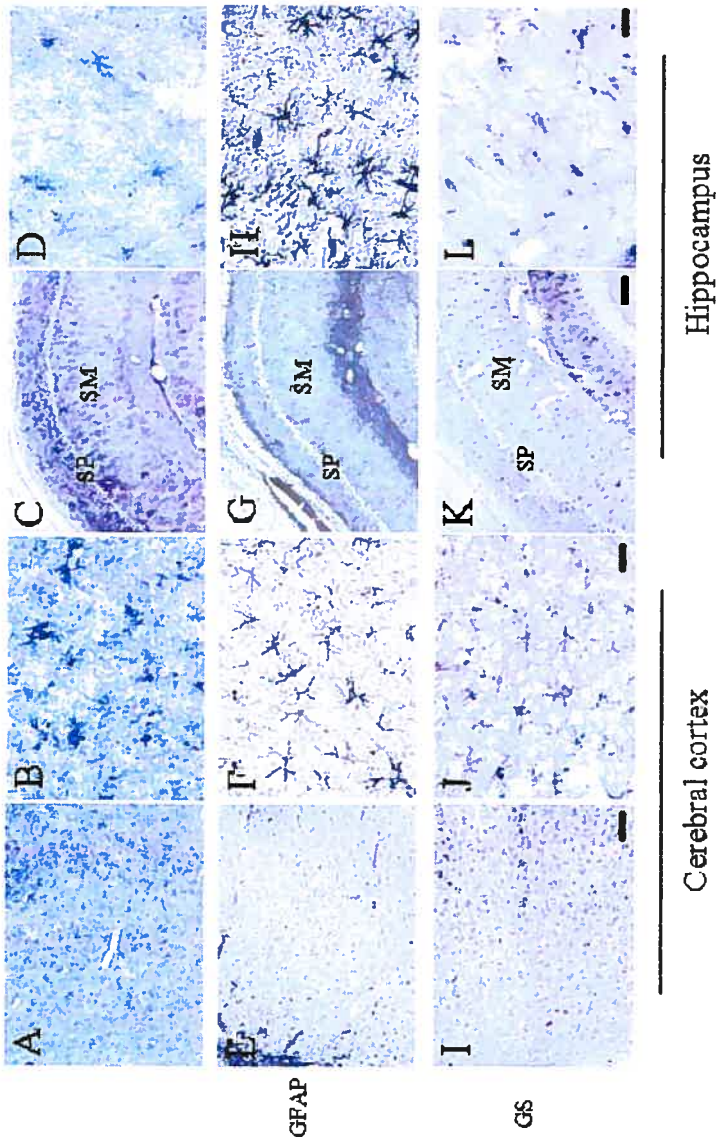

A
B
C
D
E
F
G
H
I
J
K
L
SM
SP
SM
SP
SM
SP
GFAP
GS
Cerebral cortex
Hippocampus

Figure 7.

Relationship between GLT-1v and neuronal damage in the injured cerebral cortex following lateral fluid-percussion injury. A) Correlation between GLT-1v and neuronal cell counts. A significant positive correlation (p = 0.0001) was found between GLT-1v (normalized to actin) and cell numbers. The regression line shown is $y = 0.003x + 0.02$ in which the Pearson's product-moment correlation coefficient, r = 0.78. Data are from all animals in the three groups (n = 17). B) Quantitative immunoblot analysis of the neurofilament protein α-internexin. Representative immunoblot showing α-internexin bands at 6 and 24 hr post-TBI compared to a sham control. Densitometric analysis reveals decreased levels of α-internexin at 24 hr post-injury. Lanes were loaded with equal amounts of protein in each case (20 μg). Arrow shows position and size of the migrated protein. *p < 0.05 compared to sham level (ANOVA with *post-hoc* Dunn's test). C) Correlation between GLT-1v and α-internexin. A significant positive correlation (p = 0.0358) was found between GLT-1v and α-internexin. The regression line shown is $y = 0.49x - 0.11$ in which the Pearson's product-moment correlation coefficient, r = 0.51. Data are from all animals in the three groups (n = 17).

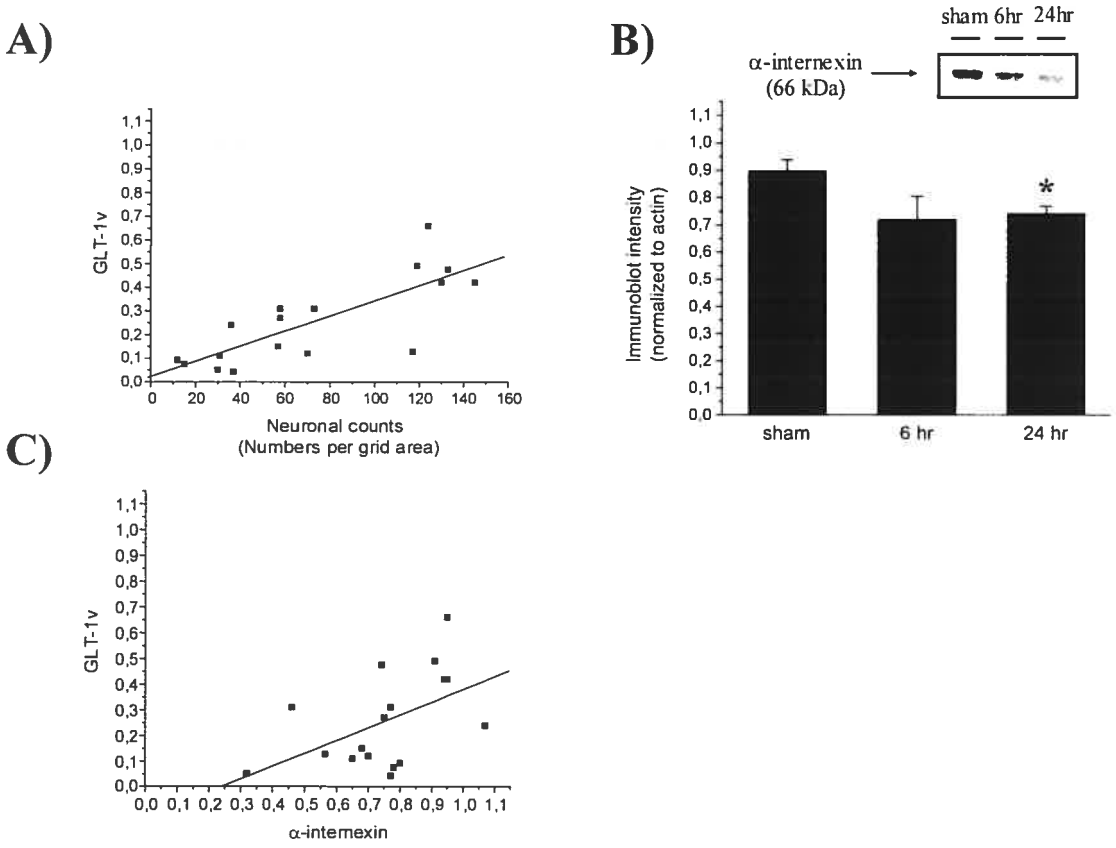
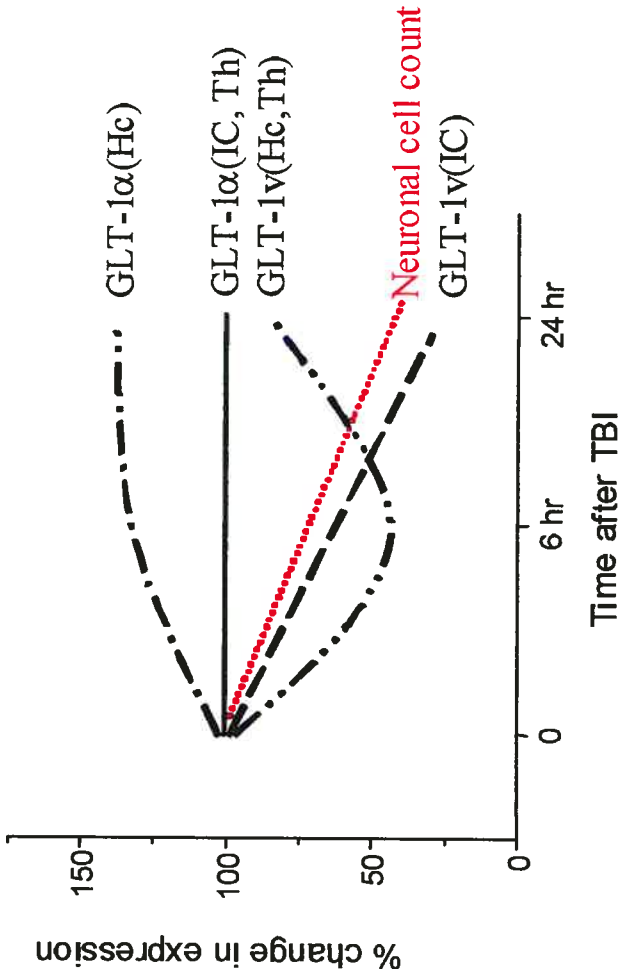

A)
GLT-1v
Neuronal counts
(Numbers per grid area)
B)
sham 6hr 24hr
α-internexin
(66 kDa)
Immunoblot intensity
(normalized to actin)
sham
6 hr
24 hr
*
C)
GLT-1v
α-internexin

Figure 8.

Graphical display summarizing the regional changes in expression levels of GLT-1α, and GLT-1v at 6 hr and 24 hr following fluid percussion injury. Decrease in GLT-1v, but not GLT-1α, is positively correlated with neuronal loss. Abbreviations: IC, injured cortex; Hc, hippocampus; Th, thalamus.


% change in expression
150
100
50
0
0
6 hr
24 hr
Time after TBI
GLT-1α(Hc)
GLT-1α(IC, Th)
GLT-1v(Hc,Th)
Neuronal cell count
GLT-1v(IC)

2.2 Article 2

Early, transient increase of complexin I and II in the cerebral cortex following traumatic brain injury is attenuated by *N*-acetylcysteine.

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ABSTRACT

Alteration of excitatory neurotransmission is a key feature of traumatic brain injury (TBI) in which extracellular glutamate levels rise. Although increased synaptic release of glutamate occurs at the injury site, the precise mechanism is unclear. Complexin I and complexin II constitute a family of cytosolic proteins involved in the regulation of neurotransmitter release, competing with the chaperone protein α-SNAP (soluble *N*-ethylmaleimide-sensitive factor-attachment protein) for binding to the synaptic vesicle protein synaptobrevin as well as the synaptic membrane proteins SNAP-25 and syntaxin which together form the SNAP receptor (SNARE) complex. Complexin I is predominantly a marker of axosomatic (inhibitory) synapses, whereas complexin II mainly labels axodendritic and axospinous synapses, the majority of which are excitatory. In order to examine the role of these proteins in TBI, we have studied levels of both complexins in the injured hemisphere by immunoblotting following lateral fluid-percussion brain injury in the rat. Transient increases in the levels of complexin I and complexin II proteins were detected in the injured cerebral cortex 6 h following TBI. These effects were completely blocked by *N*-acetylcysteine (NAC) administered 5 minutes following trauma, suggesting an involvement of oxidative stress. Neuronal loss was also reduced in the injured hemisphere with post-TBI NAC treatment. Our findings suggest a dysregulation of both inhibitory and excitatory neurotransmission following traumatic injury that is responsive to antioxidant treatment. These alterations in complexin levels may also play an important role in neuronal cell loss following TBI, and thus contribute to the pathophysiology of cerebral damage following brain injury.

Running title: Complexins in TBI

Key words: traumatic brain injury, neurotransmitter, excitotoxicity, complexin, fluid-percussion, antioxidant

INTRODUCTION

Traumatic brain injury (TBI) is a multifaceted disease. Complications such as intracranial hemorrhages and diffuse axonal injury can occur that could contribute to or superimpose upon secondary damage such as ischemia, oxidative stress, and massive edema. Such secondary injury can cause profound alterations of endogenous neurochemical processes including excitatory mechanisms that may be a major cause of neuropathological damage.

Glutamate is the major excitatory neurotransmitter in brain. In both humans and animal models of TBI, an abrupt increase in extracellular levels has been well documented (Faden et al., 1989; Katayama et al., 1990; Palmer et al., 1993; Bullock et al., 1998). Such a rise in interstitial glutamate has probably many origins; extravasation of blood into the parenchymal space (Koizumi et al., 1997), development of membrane micropores leading to release of structural amino acids (Bullock et al., 1998), glutamate uptake dysfunction due to downregulation of the astrocytic glutamate transporters GLT-1 and GLAST (Rao et al., 1998; Yi et al., 2004), and enhanced synaptic release and subsequent overflow from the synaptic terminal. Increased glutamate levels can play a major role in the development of secondary excitotoxic injury; thus understanding the exact source of post-traumatic glutamate increase is necessary in order to improve our understanding and develop potential therapeutic strategies.

Complexins are 15-16 kDa cytosolic proteins that compete with the chaperone protein α-SNAP (soluble *N*-ethylmaleimide-sensitive factor-attachment protein) for binding to SNAP receptors (SNAREs) at the presynaptic terminal (Pabst et al., 2000). These SNAREs consist of the synaptic vesicle protein synaptobrevin as well as the synaptic membrane proteins SNAP-25 and syntaxin 1 (McMahon et al., 1995; Pabst et al., 2000). Complexins have been shown to rapidly bind to the SNARE complex in an anti-parallel configuration and with high affinity (Pabst et al., 2002). However, the exact contribution of complexins to neurotransmitter release has been a controversial issue, with some studies indicating that complexins play a negative role in exocytosis,

and others suggesting a positive role in neurotransmitter release (Ono et al., 1998; Reim et al., 2001).

Notably, the localization of complexin I in inhibitory neurons and complexin II in excitatory neurons in the cerebellum and hippocampus (Takahashi et al., 1995; Ishizuka et al., 1999; Yamada et al., 1999) has permitted differential assessment of excitatory and inhibitory pathways (Eastwood et al., 2001). In psychological disorders such as major depression, bipolar disease and schizophrenia (Sawada et al., 2002; Eastwood and Harrison, 2001), complexin levels have been measured in post-mortem brain tissue in order to assess the extent of excitatory or inhibitory involvement in the region of interest. Moreover, in a transgenic mouse model of Huntington's disease, progressive loss in complexin II was detected (Morton and Edwardson, 2001), indicating the potential importance of this novel presynaptic protein in neurodegenerative disease states.

In brain trauma, the mechanisms by which increased brain glutamate and GABA levels occur following injury remain unclear. In this study, we have measured changes in complexin levels in brain regions of rat subjected to lateral fluid-percussion (FP) brain injury of moderate severity in order to determine whether TBI might alter excitatory and/or inhibitory neurotransmission via an effect on this part of the synaptic release machinery. In addition, we examined whether changes in complexin levels may be due to oxidative stress-related events, via treatment of animals with the well-known antioxidant *N*-acetylcysteine (NAC). Our results demonstrate, for the first time, that TBI results in a dynamic change in complexin I and complexin II protein levels, and that this change is likely associated with oxidative stress.

MATERIALS AND METHODS

Materials

Protease inhibitor cocktail, mouse monoclonal antiserum to β-actin, rabbit anti-goat IgG secondary antibody, 3,3'-diaminobenzidine (DAB), nickel ammonium sulfate and sodium azide were purchased from Sigma Chemicals Co (St. Louis, MO, USA). Agarose-rabbitt IgG was purchased from Sigma Aldrich (Canada).

Polyvinylidene difluoride (PVDF) membranes were purchased from Bio-Rad Laboratories (Hercules, CA, USA), broad-range protein markers were purchased from Amersham Canada Ltd (Oakville, ON, Canada), enhanced chemiluminescence kits were purchased from Perkin Elmer (Woodbridge, ON Canada), and X-OMAT autoradiography film was purchased from Kodak (Ile de Soeurs, Quebec, Canada). Complexin I and II antibodies were generous gift from Dr. Ken Sawada (Vancouver general hospital, Canada) and Seiichi Takahashi (Koichi medical school, Japan) and previously characterized (Takahashi et al., 1995). Biotinylated donkey anti-rabbit, anti-goat and anti-mouse antibodies were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA). Protein A/g-agarose conjugate was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). .Monoclonal anti-nitrotyrosine antibody was purchased from Upstate Biotechnologies (USA).

Brain injury model

All procedures were undertaken with the approval of the Animal Ethics committee of Hôpital Saint-Luc and the University of Montreal, and were conducted in accordance with the guidelines set out by the Canadian Council on Animal Care. A total of 66 male, Sprague-Dawley rats (350-400g, Charles River, QC, Canada) were housed under constant conditions of temperature, humidity, and 12:12 h day/night cycle. The lateral (parasagittal) model of traumatic brain injury used in this study has previously been described in detail (McIntosh et al., 1989). A 4.8mm diameter craniectomy, centered between bregma and lambda, and 2.5mm lateral to the superior sagittal sinus was made over the left hemisphere using a trephine drill, and a hollow female Luer-Lok fitting secured with dental cement. One group of rats was subsequently allowed to recover overnight and, the following day, reanesthetized with 2% isofluorane and subjected to lateral FP injury of moderate severity (2.0-2.5 atm, n = 52). Sham-injured controls (n = 14) received identical treatment but without exposure to trauma. Animals were allowed to recover for 6 h, 24 h, 72 h (3 days) and 168 h (7 days) post-injury prior to sacrifice. For the administration of NAC, rats were administered either antioxidant (163 mg/kg, i.p., n = 21) or an equivalent volume of saline (n = 21) 5 min following TBI. Previous study in our group showed reduced

injury volume and Heme oxygenase-I induction at this amount of NAC administration (Yi and Hazell, 2005).

Immunoblotting

Animals were anesthetized with pentobarbital (60 mg/kg, i.p.) and the brains were removed and rapidly frozen in isopentane on dry ice, sectioned into appropriate regions corresponding to the injured cerebral cortex, hippocampus and thalamus as previously described (Yi et al., 2004). Tissues were homogenized in ice cold TE buffer (50 mM Tris, 150 mM NaCl) with a protease inhibitor cocktail (Sigma Chemical Co., St. Louis, MO, USA) and phenylmethylsulfonyl fluoride using a Teflon glass homogenizer at 1000 rpm, 25 strokes, on ice. The resulting homogenate was centrifuged for 30 minutes at 15 000 x g, 4 °C, and the supernatant was separated into another tube. Protein content was determined using Bio-Rad DC Protein Assay kit (Hercules, CA, USA). Immunoblotting assays were performed as previously described (Yi et al., 2004). Briefly, 10 to 15 micrograms of tissue were boiled in equal volume of 2X Laemmli buffer with 0.1 M dithiothreitol (final concentration) for 5 min, and separated using discontinous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) consisting of 13% resolving and 4% stacking gels, separated overnight at 40 V, and then transferred to PVDF membranes at 20 V constant voltage overnight at 4°C. These membranes were subsequently probed for complexin I (1:1,000) and complexin II (1:1,000) using hybridoma-derived antibodies or for β-actin (1:20,000) using a monoclonal antibody (Sigma Chemical Co., St. Louis, MO, USA), and semi-quantitation of the intensity of immunobands was performed using a microcomputer-based image display system (Imaging Research Inc., St. Catherines, ON, Canada).

Immunoprecipitation

Four hundred micrograms of the homogenates from normal rat tissue prepared as described in the immunoblotting section were used for immunoprecipitation experiments. The samples were precleared with control IgG (agarose-rabbit IgG, Sigma-Aldrich, Canada) together with 20 µl of appropriate suspended (25% v/v)

agarose conjugate (protein A/g-agarose, Santa Cruz, USA) for 1 h at 4 °C with agitation. After brief centrifugation (1 000 x g, 30 sec, 4 °C), supernatants were transferred to new microtubes and incubated with complexin primary antibody (1:100) for 1 h at 4 °C with agitations. The primary antibody was omitted for negative control (-Ab). At the end of incubation, 20µl of appropriate agarose conjugate suspension was added and the tubes left at 4°C overnight on a rocker platform with gentle shaking. The next day, tubes were centrifuged and supernatants were discarded. Resulting pellets were resuspended in 20µl of TE buffer following washes (4 times) with 1x phosphate buffered saline. Ten µl was loaded onto SDS-PAGE gel, and ran under non-reducing condition (no DTT, no boiling) for figure 1. Rainbow wide-range molecular weight marker used to determine the approximate molecular weight of complexins was purchased from Amersham, USA.

Immunohistochemistry and Histology

For immunostaining and histology, rats were deeply anesthetized with pentobarbital (60 mg/kg) and perfused transcardially as described previously (Hazell et al., 2001). Briefly, brains were removed and post-fixed overnight in neutral-buffered formalin containing 4% formaldehyde, 0.5% sodium phosphate buffer, and 1.5% methanol, pH.7.0. Coronal sections (-3.8 to -5.8 mm relative to bregma) were cut (5 µm thickness) from paraffin-embedded blocks of tissue or with a vibrotome (40 µm thickness). Paraffin-embedded sections were stained with cresyl violet. Vibrotome-cut sections were processed as previously described (Yi et al., 2004) for complexin I and complexin II immunohistochemistry. Anti-nitrotyrosine immunohistochemstry was performed according to the manufacturer's recommended procedure. Following washing and alcohol dehydrations, the resulting sections were mounted on Superfrost Plus slides (Fisher Scientific, Ottawa, ON, Canada), cleared in xylene (Fisher Scientific, Ottawa, ON, Canada), and coverslipped with Permount (Fisher Scientific, Ottawa, ON, Canada). Neuronal cell numbers were assessed by counting and averaging of four adjacent grid areas (0.06 mm^2 each) at a magnification of 400X using an Olympus BX-51 light microscope.

Statistical analysis

Statistical analysis for the quantitative immunoblotting and neuronal counting was performed using analysis of variance (ANOVA) with *post-hoc* Tukey test ($p < 0.05$) and the Mann-Whitney *U* test ($p<0.05$).

RESULTS

FP brain injury of moderate severity caused an acute and transient increase in complexin I and II protein levels in the ipsilateral (injured) temporal-parietal cortex, detected by immunoblotting. Immunoblots showed a single distinct band (Fig. 1A) on 13 % SDS-PAGE gels for complexin I (15 kDa) and complexin II (16 kDa) under reducing condition, as previously reported (Yamada et al., 1999). For complexin I, what appears to be a dimer band also was detected when the homogenates were ran under non-reducing condition (Fig. 1B), but addition of DTT and boiling the samples before running the gel completely abolished forming of this dimer band for complexin I, resulting in appearance of single band. Immunoprecipitation and subsequent immunobotting demonstrated that the antibodies are specific to each complexin and do not cross-react between the two complexins (Fig. 1B). The transient increase was particularly evident in the case of complexin I, where the protein level increased approximately 150 % of sham level at 6 h post injury (Fig. 2A). Levels of complexin II showed a similar transient increase, but which was less pronounced, showing 50 % increase at 6h post-TBI (Fig. 2A). This increase was only seen in the injured cortex, and not other regions studied such as hippocampus (Fig. 2B) and thalamus (Fig. 2C). Interestingly, a small but significant decrease of complexin I level was detected at 3 and 7 days following trauma in the injured cortex (42 % at day 3, 44 % at day 7) and 7 days following trauma in hippocampus (31 % at day 7), suggesting diminished inhibitory input at these time points in the regions (Fig. 2A, B). In the injured thalamus, both complexin I (40 % at day 3 and 43 % at day 7) and complexin II (47 % at day 3 and 46 % at day 7) showed a significant decrease from the sham level following the injury (Fig. 2C). Immunohistochemical studies revealed that numerous complexin I stained neurons were particularly evident in layers 3-5 of cerebral cortex

(Fig. 3Ai-iii), with several complexin I immunoreactive neurons in the CA1-3 hippocampal subfields (Fig. 3Aiv-vi). In the cerebral cortex, numerous small neurons in layers 5-6 showed intense complexin II immunoreactivity (Fig. 3Avii-ix).

We used nitration of tyrosine residues as a qualitative assessment of amount of oxidative damage in the injured cerebral cortex following trauma. Relatively low number of cells was immunopositive for Anti-NT antibody in the cerebral cortex of sham-injured rats (Fig. 3Bi). Six hours after the insult, large number of cells showed positive immunoreactivity for Anti-NT in the adjacent cerebral cortex (Fig. 3Bii), and NAC treatment partially reduced the amount of immunopositive cells (Fig. 3Biii). When compared using light microscopy, Anti-NT positive neurons shared morphological similarity with complexin I or II-immunopositive neurons in the cerebral cortex (Fig. 3C).

Figure 4 shows the results of studies that investigated whether these increases in complexin levels following TBI were due to oxidative injury-related events by treatment with NAC. Histological assessment with cresyl violet indicated that the NAC-treatment produced a 34%, 34%, and 55% reduction in neuronal loss in the injured cerebral cortex, hippocampus, and thalamus respectively relative to the contralateral hemisphere 24 h post-TBI when compared to the corresponding saline-treated group (Table 1, Fig. 4). In addition, NAC treatment completely abolished the transient increase in complexin I and complexin II levels observed at 6 h in the injured cerebral cortex in saline-treated TBI groups (Fig. 5A-C). Levels of complexin I in the injured cortex of NAC-treated rats also displayed a further decrease at 24 h when compared to levels in the cortex of non-NAC-treated TBI animals (Fig. 5A,B).

DISCUSSION

Brain trauma caused a significant increase in the levels of complexin I and complexin II in the injured cerebral cortex at 6h post-injury, and this change was an acute and transient event. At later time points (day 3 and day 7 after the insult), complexin I/complexin II ratio decreased, suggesting reduced inhibitory input in the injured cortex and also in hippocampus region. In this model of TBI, neuronal loss is

most severe in the cortex; thus, these complexin changes likely reflect an alteration in synaptic activity that could contribute to the neuronal damage. Our immunohistochemical findings revealed a differing pattern of complexin I and complexin II expression, each being present in different layers of cerebral cortex which is in line with previous reports (Yamada et al., 1999). As complexin I is localized in axo-somatic synapses and complexin II in axo-dendritic and axo-spinous synapses (Takahashi et al., 1995; Ishizuka et al., 1999) that are predominantly inhibitory and excitatory in nature, respectively (Peters and Palay, 1996), altered levels of both complexins likely indicate a dysregulation of both inhibitory and excitatory neurotransmitter release in the injured cortex.

In the present study, we tested the effect of antioxidant treatment on complexin levels and on overall neuronal loss following TBI. Use of the well-known antioxidant NAC has previously been shown to restore the vascular responsiveness to vasomodulators following midline FP injury in cats (Ellis et al., 1991), to reduce the decrease in mitochondrial activity following cortical impact injury in rats (Xiong et al., 1999), and to exert a potent neuroprotective effect in focal cerebral ischemia, also in rodents (Sekhon et al., 2003). Our findings indicate that NAC treatment completely reversed the increase in complexin protein levels at 6 h in the injured cortex following TBI to sham levels. Recently, we reported that NAC administered 5 min post-injury produced a significant reduction in the volume of injury in rats subjected to lateral FP (Yi and Hazell, 2005). In the present study, we have demonstrated that neuronal loss following TBI was diminished in NAC-treated rats in all three regions studied. These results suggest that oxidative stress plays an important role in the histological damage evident in the cortex, and that alterations in excitatory and inhibitory neurotransmitter release may be important contributing factors.

Our understanding of the role of complexins has been limited thus far. Complexins may either inhibit or promote vesicular release of neurotransmitters, with conflicting reports of their function. Studies showing inhibition of vesicular release by overexpression of complexins in PC12 cells or the injection of complexin antibodies in squid giant axon indicate that complexins may have an inhibitory effect on neurotransmitter release (Itakura et al., 1999; Ono et al., 1998). On the other hand, a

double mutant deletion study has shown a reduction in vesicular release (Reim et al., 2001), and microinjection of a partial complexin peptide sequence that competes with the binding of complexin to syntaxin completely inhibited neurotransmitter release at later fusion steps (Tokumaru et al., 2001), together suggesting that complexins are positive regulators of neurotransmitter release.

Regardless of whether complexins may block or enhance neurotransmitter release prior to or following neurotransmitter vesicle fusion with the plasma membrane, it seems likely these proteins are a part of the neurotransmitter release machinery. In a recent study, Abderrahmani et al (2004) have shown that both knocking down or overexpression of complexin I in pancreatic beta-cells using RNA interference or overexpression via transfecting vectors expressing the complexin I gene leads to strong impairment in beta-cell secretion. Complexins may be involved in rapid closure and recycling of fusioned membranes since overexpression of complexin II leads to a reduced half-life of synaptic vesicle release events (Archer et al., 2002). Based on these two findings, complexins may positively regulate synaptic vesicle fusion. A decrease in complexin availability could result in a reduction in the number of vesicular fusion events, thus lowering the amount of vesicular release. Also, overexpression of complexins may result in rapid closure and recycling of vesicles leading to premature and incomplete termination of the release event, again limiting the amount of vesicular release. In general, it would seem that an adequate level of these regulatory molecules is necessary for the maintenance of functional synaptic events (Abderrahmani et al., 2004), though the exact function of complexins remains uncertain.

In summary, our findings of an early transient increase of both complexins may reflect a change in synaptic activity in the injured cerebral cortex, particularly at inhibitory synapses, since complexin I levels changed more dramatically. Oxidative stress may manifest its effect partly via complexin changes, leading to neuronal loss, with NAC having an ability to reduce the extent of this effect. At present, the exact contribution(s) of complexins to synaptic vesicle release and to the amount of neurotransmitter released in the injured cortex of rats following TBI remains undetermined. However, very recent expression studies involving complexin I and

complexin II localization indicate that the functional role of these proteins may be based not only on the identity of the neurotransmitter, but also upon the associated neuronal circuitry (Freeman and Morton, 2004). Thus, future studies will be required to better understand the consequences of changes in complexin levels following TBI.

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Table 1. Neuronal counts in the injured cerebral cortex, hippocampus, and thalamus 24 h following TBI in the absence or presence of NAC treatment.

	Saline + TBI		NAC + TBI	
	Ipsilateral	Contralateral	Ipsilateral	Contralateral
Cerebral Cortex	$7 \pm 3^{*\dagger}$	61 ± 5	$25 \pm 5^{*}$	55 ± 4
Hippocampus	$11 \pm 3^{*\dagger}$	34 ± 2	$22 \pm 3^{*}$	33 ± 1
Thalamus	$8 \pm 1^{*\dagger}$	20 ± 3	17 ± 1	18 ± 1

Values are mean ± SEM. $^{*}p < 0.01$ compared to contralateral side of same group (ANOVA with *post-hoc* Tukey test). $^{\dagger}p < 0.05$ compared to ipsilateral side of NAC-treated group (ANOVA with *post-hoc* Tukey test).

Figure 1.

Complexin I (Cpx I) and complexin II (Cpx II) are distinct proteins abundantly present in brain homogenates. (A) Representative western blot bands for Cpx I (white arrowhead) and Cpx II (black arrowhead) in which forebrain homogenate (5 μg per lane) was loaded onto SDS-PAGE gel (13%) and electrophoresed under non-reducing condition, transferred onto PVDF membranes and probed for Cpx I (left lane) or Cpx II (right lane). (B) Immunoprecipitation products (10 μl) for Cpx I (IP-cI) and Cpx II (IP-cII) were loaded onto SDS-PAGE gel (13%) and electrophoresed under non-reducing conditions along with immunoprecipitation control (-AB, no antibody), subsequently transferred, and probed with either Cpx I antibody (left 3 lanes) or Cpx II antibody (right 3 lanes). Note that complexin antibodies do not cross-react with each other.

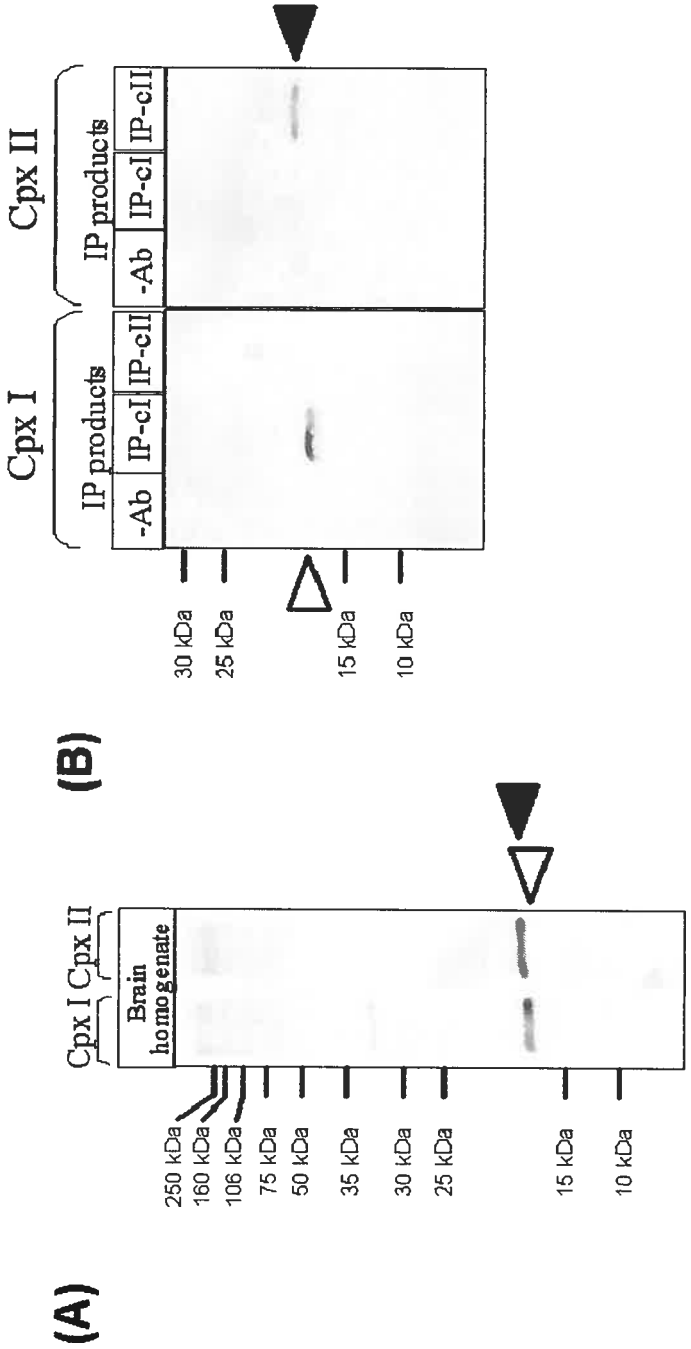

(A)
Cpx I Cpx II
Brain homogenate
250 kDa
160 kDa
106 kDa
75 kDa
50 kDa
35 kDa
30 kDa
25 kDa
15 kDa
10 kDa
(B)
Cpx I
Cpx II
IP products
-Ab
IP-cI
IP-cII
30 kDa
25 kDa
15 kDa
10 kDa

Figure 2.

Complexin I (Cpx I) and complexin II (Cpx II) levels undergo dynamic changes following moderate FP injury. Percent optical density (normalized to actin levels) of Cpx I and II in the injured cortex (Ctx) (A), hippocampus (Hc) (B), and thalamus (Thal) (C) at 5 different time points following trauma. Insets; representative immunoblots of appropriate region for Cpx I, Cpx II and β-actin in sham, 6 h, 24 h, 3 days and 7 days following TBI. Note that in the injured cortex Cpx I levels increased dramatically at 6h following injury, while Cpx II levels increased to a lesser extent. At day 3 and 7 in the injured cortex and hippocampus, Cpx I levels decreased but not that of Cpx II. In the injured thalamus, both complexins showed decreased levels at day 3 and 7 following trauma. Values are converted to percent sham (sham value = 100 %) ± S.E.M. *$p < 0.05$ compared to sham value (ANOVA with post-hoc Tukey test).

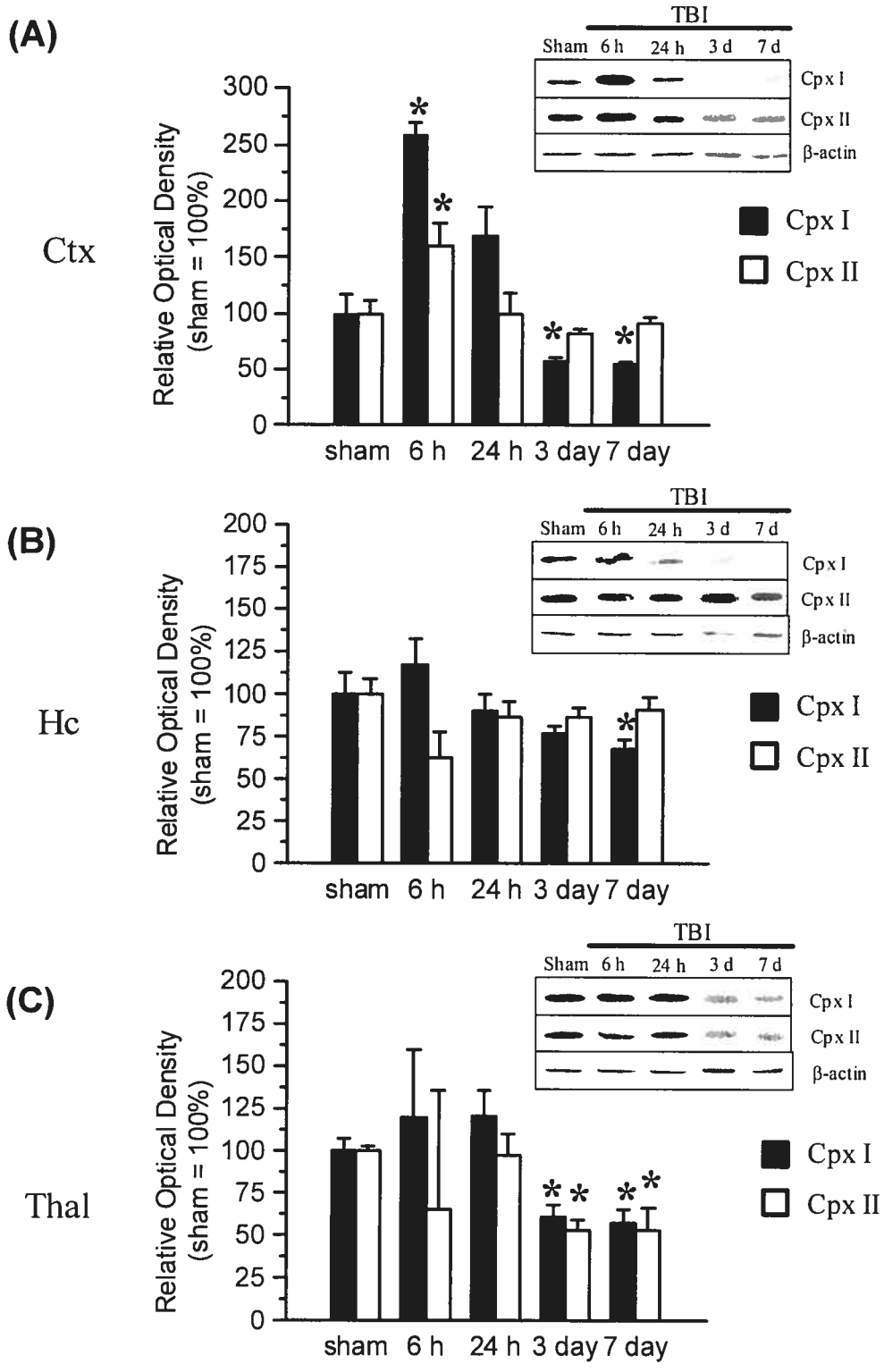

(A)
Ctx
TBI
Sham 6 h 24 h 3 d 7 d
Cpx I
Cpx II
β-actin
Relative Optical Density (sham = 100%)
300
250
200
150
100
50
0
sham 6 h 24 h 3 day 7 day
Cpx I
Cpx II
(B)
Hc
TBI
Sham 6 h 24 h 3 d 7 d
Cpx I
Cpx II
β-actin
Relative Optical Density (sham = 100%)
200
175
150
125
100
75
50
25
0
sham 6 h 24 h 3 day 7 day
Cpx I
Cpx II
(C)
Thal
TBI
Sham 6 h 24 h 3 d 7 d
Cpx I
Cpx II
β-actin
Relative Optical Density (sham = 100%)
200
175
150
125
100
75
50
25
0
sham 6 h 24 h 3 day 7 day
Cpx I
Cpx II

Figure 3.

(A) Immunohistochemical staining of complexin I (Cpx I) and complexin II (Cpx II) in the cerebral cortex and hippocampus. In the cortex, numerous Cpx I immunoreactive neurons were observed (i-iii), with the highest number of neurons present in layers 2-3 and 4-5. In the hippocampus, several scattered neurons displayed Cpx I immunoreactivity (iv-vi). Numerous small Cpx II immunoreactive neurons were also found scattered in layers 5 and 6 of the lateral cortex (vii-ix). Bars, 400 μm (i, iv, vii); 80 μm (ii, v, viii); 40 μm (iii, vi, ix). (B) Qualitative assessment via immunohistochemical comparison of nitration of tyrosine residue in proteins using anti-nitrotyrosine (anti-NT) antibody following sham injury (i), 6 h TBI with saline injection (ii), and 6 h TBI with N-acetylcysteine (NAC). Injection of NAC reduced neurons positive for nitrotyrosine staining at 6 h following TBI in the injured cortex. Bar, 200 μm. (C) Morphological comparison of anti-NT-positive neurons at 6 h following the injury (i) compared to Cpx I (ii) and Cpx II (iii) in the cortex of sham rats. Bar, 80 μm.

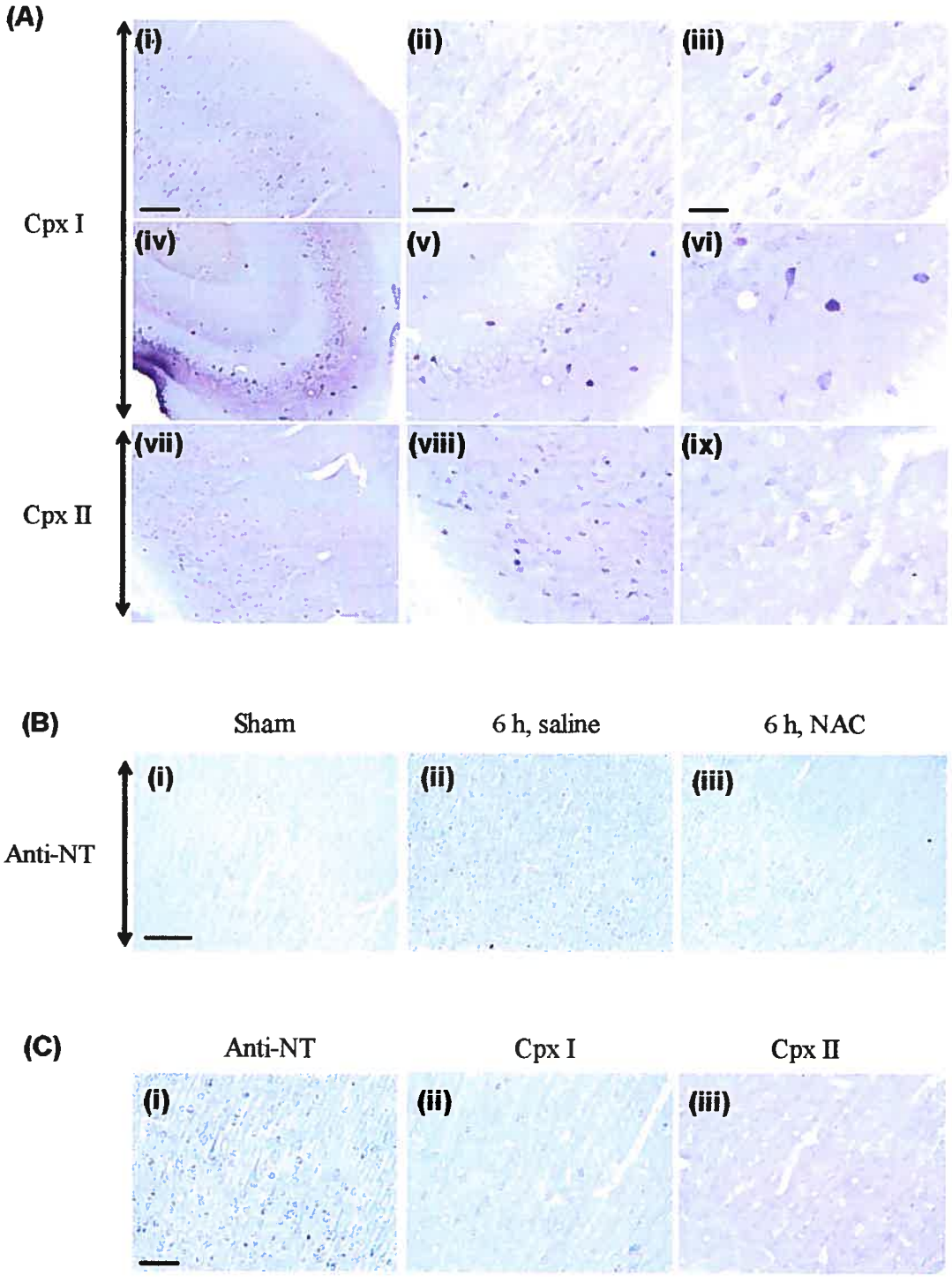

(A)
Cpx I
Cpx II
(i)
(ii)
(iii)
(iv)
(v)
(vi)
(vii)
(viii)
(ix)
(B)
Sham
6 h, saline
6 h, NAC
Anti-NT
(i)
(ii)
(iii)
(C)
Anti-NT
Cpx I
Cpx II
(i)
(ii)
(iii)

Figure 4.

Cresyl violet staining in animals treated with saline or NAC 24 h following TBI. NAC treatment reduced neuronal cell loss 24 h following the insult in injured cerebral cortex (Ctx) (A-C), hippocampus (Hc) (D-F) and thalamus (Thal) (G-I). Panels A, D, and G show saline-treated rats 24 h post-TBI. Panels B, E, and H show NAC-treated rats 24 h post-TBI. Panels C, F, and I show the contralateral hemisphere to the saline-treated rat 24 h following TBI. Bar, 50 μm.

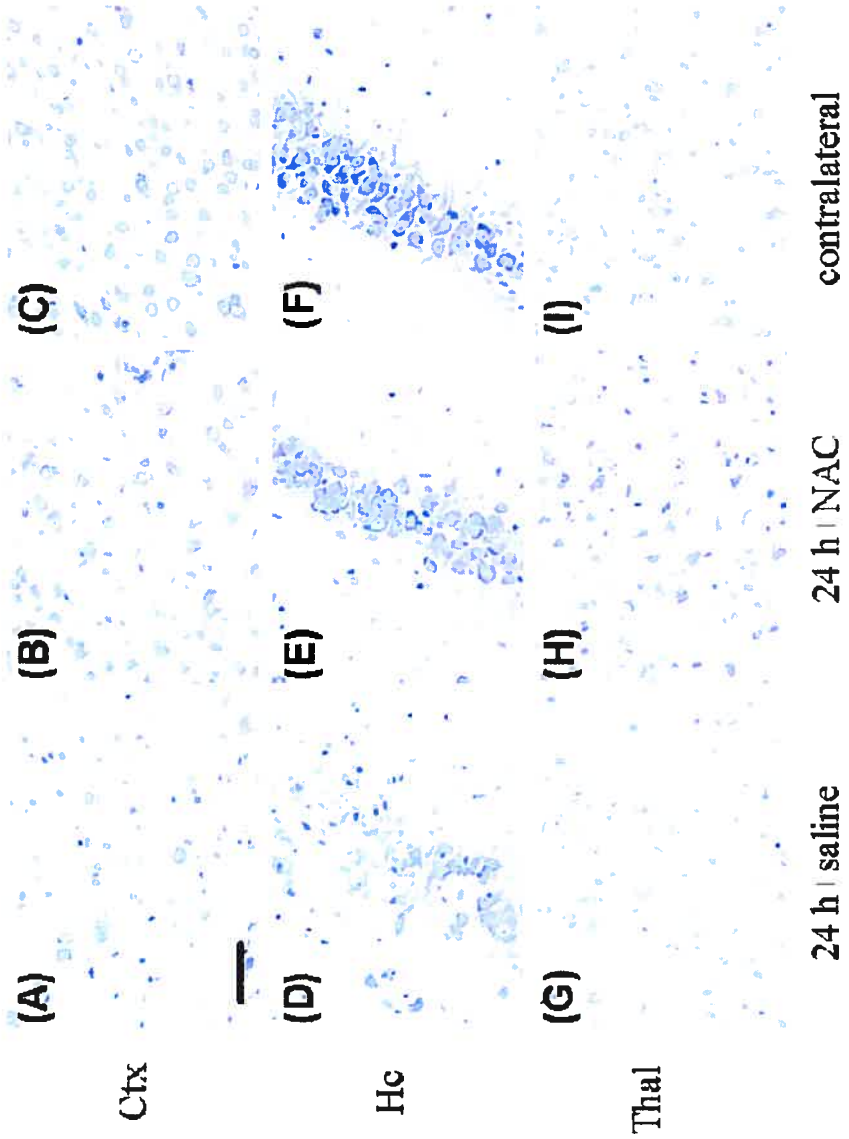
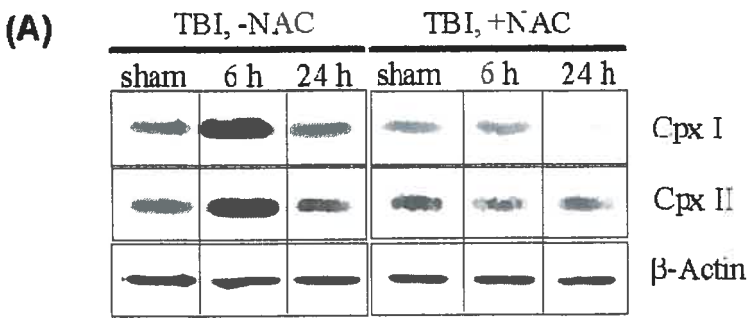
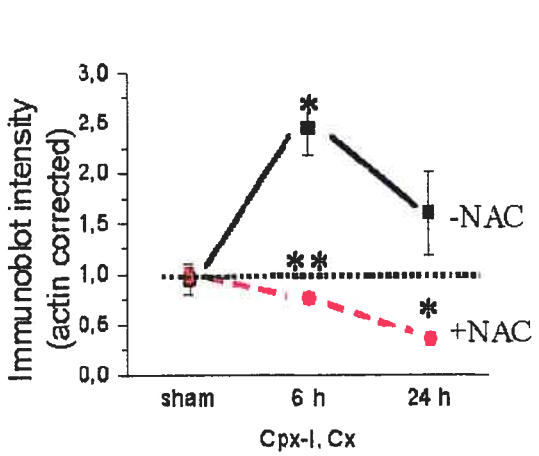
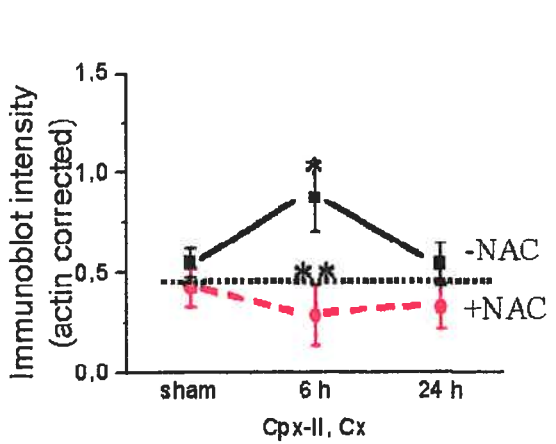

(A)
(B)
(C)
(D)
(E)
(F)
(G)
(H)
(I)
Ctx
Hc
Thal
24 h | saline
24 h | NAC
contralateral

Figure 5.

Effects of the antioxidant NAC on complexin levels in cerebral cortex following TBI. (A) Representative bands for complexin I (Cpx I), complexin II (Cpx II), and β-actin in sham-treated, 6 h and 24 h post-TBI groups, in the absence or presence of NAC treatment 5 min following the injury. (B) and (C) Immunoblot analysis; broken line: NAC-treated, solid line: saline-treated. Transient rise in Cpx I and Cpx II levels following trauma was blocked by a single injection of NAC (163 mg/kg, i.p.). Values are mean ± SEM. *$p < 0.05$ compared to sham-treated group. (ANOVA with *post-hoc* Tukey test). **$p < 0.05$ compared with same time point of the saline (-NAC)-treated group (Mann-Whitney *U* test).


(A)
TBI, -NAC
TBI, +NAC
sham
6 h
24 h
sham
6 h
24 h
Cpx I
Cpx II
β-Actin


(B)
Immunoblot intensity
(actin corrected)
3,0
2,5
2,0
1,5
1,0
0,5
0,0
-NAC
+NAC
sham
6 h
24 h
Cpx-I, Cx


(C)
Immunoblot intensity
(actin corrected)
1,5
1,0
0,5
0,0
-NAC
+NAC
sham
6 h
24 h
Cpx-II, Cx

2.3 Article 3

Glutamate transporter EAAT4 is increased in hippocampal astrocytes following fluid-percussion injury in the rat.

Submitted to Brain Research

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ABSTRACT

Functional impairment of glutamate transporters contributes to excitotoxic damage and exacerbation of injury in certain neurodegenerative disorders. Several high affinity sodium-dependent glutamate transporters have been cloned thus far. Of these, EAAT4 is abundantly expressed in Purkinje cells of the cerebellum in rats. However, little is currently known regarding levels of EAAT4 following traumatic brain injury (TBI). In this study, EAAT4 changes were examined for up to 7 days following moderate fluid-percussion by immunoblotting and immunohistochemistry. TBI caused a 20% and 25 % increase in EAAT4 levels in the injured hippocampus at day 3 and day 7 following the insult. Immunohistochemical analysis revealed this increase to be localized in cells exhibiting morphological characteristics of astrocytes. In addition, increased EAAT4 immunoreactivity was observed in astrocytes in the ipsilateral cortex, hippocampus and thalamus at day 3 post-injury, which persisted up to 7 days following the insult. Given the reported novel characteristics of chloride conductance displayed by this transporter, our findings of increased EAAT4 levels suggest this protein may play an important role in the pathophysiology of TBI.

Running title: EAAT4 and brain trauma

Key words: traumatic brain injury, glutamate transporter, excitotoxicity, astrocyte, fluid-percussion, brain trauma

INTRODUCTION

Glutamate is the most abundant excitatory neurotransmitter in the brain. It is not metabolized by extracellular enzymes, but removed from the extracellular space by reuptake, in which glutamate transporters play a crucial role. The glutamate transporters GLT-1 and GLAST have been shown to be key players in glutamate clearance by gene knockout and knockdown studies (Danbolt, 2001; Rothstein et al., 1996; Tanaka et al., 1997), with localization studies having placed them in the astrocytic compartment in brain (Lehre et al., 1995). The neuronal glutamate transporter EAAC-1 has been reported to be less of a contributor to the removal of extracellular glutamate, owing to its high extrasynaptic localization. However, information is currently lacking regarding additional members of the sodium-dependent high-affinity glutamate transporters in the forebrain region.

Under pathological conditions, e.g. following traumatic brain injury (TBI) or ischemic stroke, surges in extracellular glutamate levels can lead to excitotoxic injury via glutamate receptor overstimulation-mediated mechanisms, if not regulated tightly at the synaptic level. Thus, maintenance of glutamate transporter function is crucial following such insults to the brain. However, recent findings suggest that glutamate transport may be impaired at an early stage following trauma due to decreased expression (Rao et al., 1998; van Landergham et al., 2001; Yi et al., 2005), and antisense knockdown of GLAST and GLT-1 have been shown to exacerbate brain injury following TBI (Rao et al., 2001), supporting this notion.

The glutamate transporter EAAT4 is a member of sodium-dependent high-affinity family of glutamate transporters that also possesses chloride channel properties and is highly expressed in dendritic spines of Purkinje cells in the cerebellum (Dehnes et al., 1998). Recently, its expression has been reported in astrocytes of mouse forebrain and spinal cord (Hu et al., 2003), and in retinal astrocytes of the rat (Ward et al., 2004). To date, however, little information exists concerning EAAT4 levels in pathological conditions such as head trauma. In this study, we have therefore examined EAAT4 protein levels in rat brain following lateral fluid-percussion injury of moderate severity.

MATERIALS AND METHODS

Trauma model

All procedures were undertaken with the approval of the Animal Ethics Committee of Hopital Saint-Luc and the University of Montreal, and were conducted in accordance with the guidelines set out by the Canadian Council on Animal Care. A total of 28 male, Sprague-Dawley rats (350-400 g) (Charles River, Saint-Constant, QC, Canada) were housed under constant conditions of temperature, humidity, and 12:12 h day/night cycle. Rats were prepared for trauma as previously described (Yi et al., 2005). Briefly, animals were anesthetized using sodium pentobarbital (65 mg/kg, i.p.), and a 4.8 mm diameter craniectomy, centered between bregma and lambda, and 2.5 mm lateral to the superior sagittal sinus, was made over the left hemisphere using a trephine drill, and a hollow female Luer-Lok fitting secured with dental cement. Rats were fasted overnight prior to this surgical implantation. Animals were subsequently reanesthetized the following day with 2% isofluorane and exposed to lateral fluid-percussion injury of moderate severity (2.0-2.5 atm). Sham controls received identical treatment but without exposure to trauma.

Immunoblotting and immunohistochemistry

Following TBI, animals were sacrificed by decapitation, the brains were removed and rapidly frozen in 2-methylbutane on dry ice, appropriate regions corresponding to the injured cerebral cortex, hippocampus and thalamus dissected out and homogenized in ice cold Tris-EDTA (TE) buffer with phenylmethylsulfonyl fluoride (PMSF) and a protein inhibitor cocktail (Sigma Aldrich, Oakville, ON, Canada) using a Teflon glass homogenizer on ice. Resulting homogenates were centrifuged for 30 min at 15,000 x g, 4°C. Pellets were washed with TE buffer, recentrifuged, the supernatant discarded, and the remaining pellet solubilized in modified RIPA buffer containing (50 mM Tris, 150 mM NaCl, 2% SDS, 1% NP-40, 0.5% sodium deoxycholate, pH 8.0, with PMSF and protease inhibitor cocktail. Protein content was determined using a protein assay kit (Bio-Rad Laboratories,

Mississauga, ON, Canada). Samples were loaded onto 10% SDS polyacrylamide gels, separated overnight, and transferred to polyvinylidene difluoride membranes at 20 V, 4°C. Membranes were subsequently probed using a rabbit polyclonal antibody directed against EAAT4 (D537, 0.4 μg/ml; generous gift from Dr. Niels C Danbolt, University of Oslo, Norway), and a mouse monoclonal antibody against β-actin according to the manufacturer's recommendations (Sigma Aldrich, Oakville, ON, Canada). Semi-quantitation of band intensities was performed using a microcomputer imaging system (MCID, Imaging Research Inc., St Catherines, Ontario, Canada).

For immunohistochemistry, rats were deeply anesthetized with pentobarbital (60 mg/kg) and perfuse-fixed transcardially as described previously (Hazell et al., 2001). Brains were removed and post-fixed overnight in 10% neutral-buffered formalin (ACP Chemicals, Inc., Montreal, QC, Canada). Coronal sections of 40 μm thickness (-3.8 to -5.8 mm relative to bregma) were cut using a vibratome based on the rat brain atlas of Paxinos and Watson (1998). Immunohistochemical assessment of floating sections was performed according to Hazell and colleagues (2001). Briefly, sections were incubated for 10 min in phosphate-buffered saline (PBS) containing 0.3% hydrogen peroxide to block endogenous peroxidase activity. Tissue sections were then washed in PBS (3 x 10 min), blocked for 20 min in PBS containing 5% donkey serum or 1% bovine serum albumin, followed by incubation in blocking solution with rabbit anti-EAAT4 antiserum (D537, 4 μg/ml) for 18 hr at 4°C. The following day, sections were washed again in PBS (3 x 10 min) and incubated for one hour with biotinylated donkey anti-rabbit IgG (1:100) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) in PBS, followed by washing in PBS (3 x 10 min) and 1 hr incubation in streptavidin-HRP conjugate (1:100) (Sigma Aldrich, Oakville, ON, Canada). Sections were then washed in PBS (3 x 10 min), and then incubated with 3,3'-diaminobenzidine (0.05%) containing 25 mg/ml nickel ammonium sulfate for signal enhancement in the presence of H_2O_2 (0.03%) for 2-10 min to allow color development. Following subsequent washings, the sections were mounted on Superfrost Plus slides (Fisher Scientific Co., Ottawa, ON, Canada), dehydrated in serial alcohols, cleared in xylene, and coverslipped with Permount. Controls for

immunostaining specificity consisted of omission of the primary or secondary antibody, resulting in a subsequent loss of immunoreactivity.

RESULTS

Figure 1 shows EAAT4 protein levels determined from both immunoblotting and immunohistochemistry. Transporter levels were very high in the cerebellum of control rats as previously reported (Furuta et al., 1997). The ~65 kDa EAAT4 immunoblot monomer band for cerebellum was at least 20 times more intense than that of the hippocampus (Fig. 1A). Immunohistochemical assessment also confirmed this protein expression in the cerebellum, in which Purkinje cells and numerous cells of the molecular layer were labeled for EAAT4 (Fig. 1B, panel i), with more scattered immunostaining in the granule cell layer. In the forebrain region, staining of neuronal cell bodies and astrocytes was observed throughout cerebral cortex, in the hippocampus and in the thalamus of sham animals (Fig. 1B, panels ii, iii and iv, respectively).

Next, we investigated changes in EAAT4 protein levels in the hippocampus following TBI. Figure 2A shows representative immunoblots for EAAT4 and β-actin in the hippocampus at 3 and 7 days following the insult, compared to that of shams. TBI caused increases in EAAT4 band intensity of 20% and 25% at day 3 and day 7 following the insult, respectively ($p < 0.05$) (Fig. 2B). Immunohistochemical analysis revealed that the EAAT4 increase in hippocampus following trauma is predominantly localized in astrocytes (Fig. 3). Increased astrocytic EAAT4 staining was also observed in the injured core region and glia limitans of the cerebral cortex (Fig. 4), and in the internal capsule and, to a lesser extent, thalamic nuclei (not shown). These increases in EAAT4 levels were not detected at earlier time points (6 h and 24 h) following the insult in any region.

DISCUSSION

EAAT4 has been cloned in human and rat brain (Fairman et al., 1995; Lin et al., 1998), and immunohistochemical studies have determined it to be localized primarily in neurons, particularly in Purkinje cells and in the molecular layer of the

cerebellum (Dehnes et al., 1998; Fairman et al., 1995; Nagao et al., 1997). However, more recent studies have identified EAAT4 to be also present in astrocytes of forebrain and spinal cord (Hu et al., 2003). In this study, we confirm that several astrocytes do indeed express EAAT4 in cerebral cortex, hippocampus and thalamus of normal rats at relatively low levels, an observation in line with previous findings (Furuta et al., 1997; Hu et al., 2003). Moreover, EAAT4 levels are increased in astrocytes, but not neurons, of hippocampus at 3 and 7 days following TBI. To our knowledge, this is the first study to show an upregulation of astrocytic EAAT4 levels in the rat forebrain under such conditions.

We previously reported that fluid-percussion injury of moderate severity caused a loss of the novel glutamate transporter splice-variant GLT-1v in injured cerebral cortex (Yi et al., 2005), and this decrease persisted for up to 7 days in cerebral cortex following the insult. It is therefore possible that our present findings of an EAAT4 increase in astrocytes may represent a compensatory response to this loss of GLT-1v, with the K_m for EAAT4 reported to be approximately 10 times less than other sodium-dependent glutamate transporters (Fairman et al., 1995), indicating a higher affinity.

In the present study, EAAT4 immunostaining was also found to be present in perivascular astrocytes in injured cerebral cortex following TBI, showing a resemblance to the pattern reported for EAAT4 in the retina (Ward et al., 2004). This close relationship between EAAT4 and blood vessels suggests a specialized vascular-related function for this transporter, apart from its contribution to clearance of synaptic glutamate following the injury; a role in blood-brain barrier regulation is one such possibility.

EAAT4 displays a high chloride conductance that is not coupled to the glutamate uptake, a property different to other sodium-dependent glutamate transporters (Fairman et al., 1995). This uncoupled chloride conductance has been hypothesized to play a role in dampening cellular excitability during the electrogenic process of glutamate uptake (Wadiche et al., 1995), along with the prevention of a reduction in the rate of transport that would otherwise occur with electrogenic uptake (Eliasof et al., 1996), by switching between two modes of function, namely glutamate

transport and chloride channel activity (Wadiche et al., 1998). Thus, the EAAT4 increases observed in our study may represent a protective mechanism for astrocyte function following TBI. It will be interesting to determine if other chloride channels such as those associated with GABA-A receptors are altered in function at this stage following TBI. In addition, further studies on the function of EAAT4 are required to clarify the exact significance of the EAAT4 increases following TBI.

ACKNOWLEDGMENT

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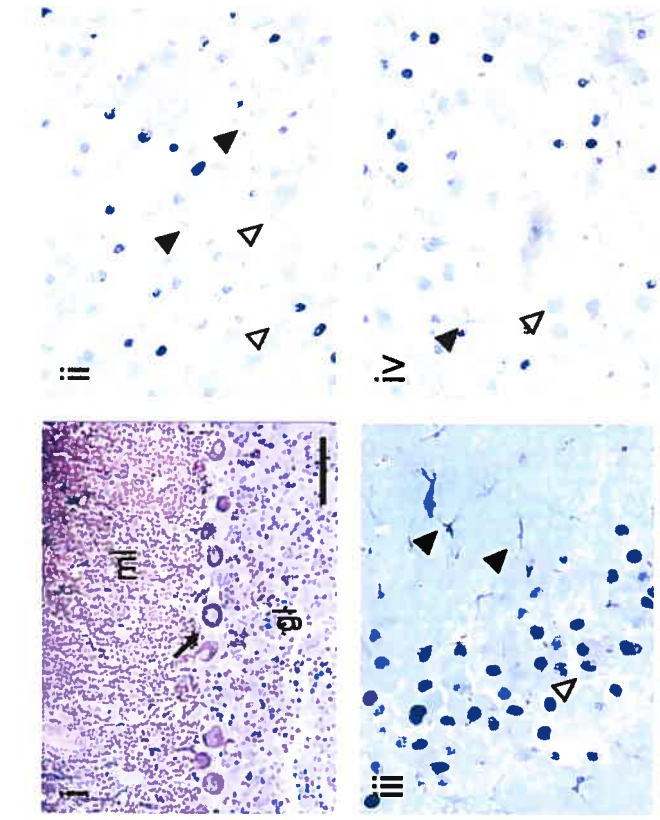
Figure 1.

Comparison of EAAT4 protein expression in rat cerebellum and forebrain regions. (A) Representative immunoblot bands for EAAT4 in cerebellar tissue (cbm, 5 μg loaded) and hipppocampus tissue (Hc, 50 μg loaded), analyzed by 10 % SDS-PAGE. (B) Immunohistochemical analysis of EAAT4 expression in cerebellum (i), cerebral cortex (ii), hippocamus (iii) and thalamus (iv). Arrow in B (i) points to cerebellar Purkinje cells darkly stained for EAAT4. Both neuronal (white arrowheads) and astrocytic (black arrowheads) staining was observed in cerebral cortex, hippocampus, and thalamus. ml; molecular layer. gl; granule cell layer. Bar; 50 μm.


(B)
ml
gl
ii
iii
iv

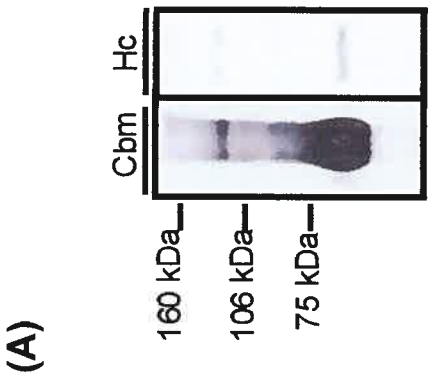

(A)
Cbm
Hc
160 kDa
106 kDa
75 kDa

Figure 2.

Hippocampal EAAT4 is increased at days 3 and 7 following TBI. (A) Representative immunoblot bands for EAAT4 in the injured hippocampus following sham-injury (S), 3 days (d3) and 7 days (d7) following moderate fluid-percussion injury in rats. Fifty micrograms of tissue were loaded onto 10 % acrylamide gel, transferred overnight and probed with anti-EAAT4 polyclonal antibody. (B) EAAT4 immunoblot band intensity normalized to β-actin showed a modest but significant 20 % and 25 % increase at day 3 and 7 following trauma, respectively, relative to sham level (asterisks, Tukey test, $p<0.05$).

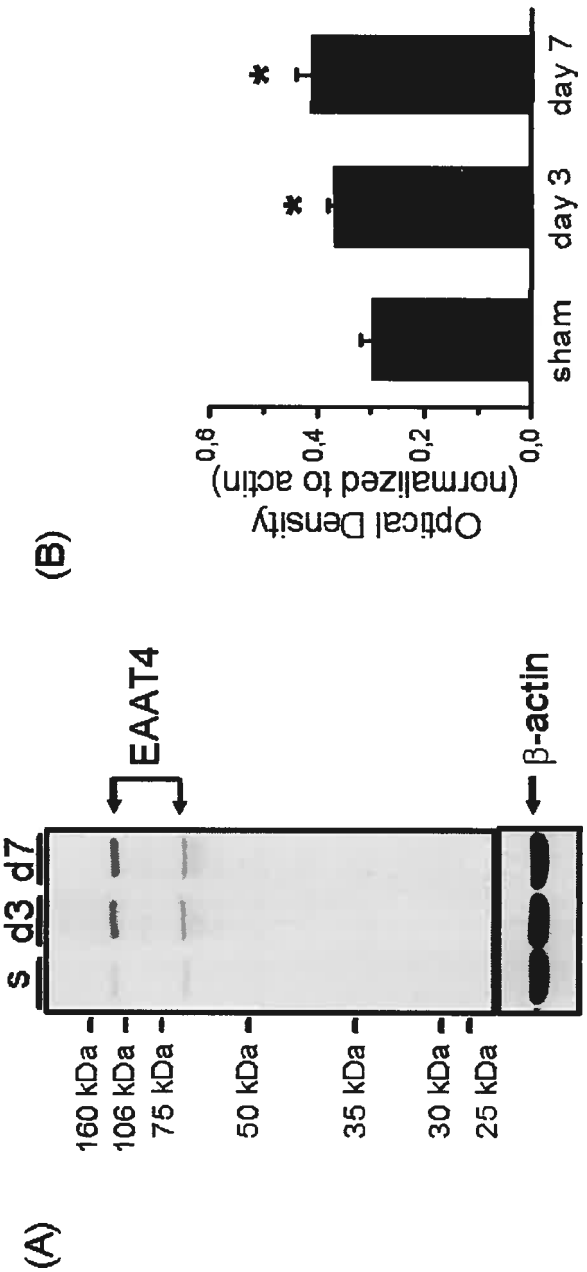

(A)
s d3 d7
EAAT4
β-actin
160 kDa
106 kDa
75 kDa
50 kDa
35 kDa
30 kDa
25 kDa
(B)
Optical Density
(normalized to actin)
0,0
0,2
0,4
0,6
sham
day 3
day 7
*
*

Figure 3.

EAAT4 immunohistochemistry showing increased astrocytic staining throughout hippocampal region at day 3 (B, E) and 7 (C, F) following TBI compared to sham (A, D). Bar; 200 μm for A, B, and C, and 50 μm for D, E, and F.

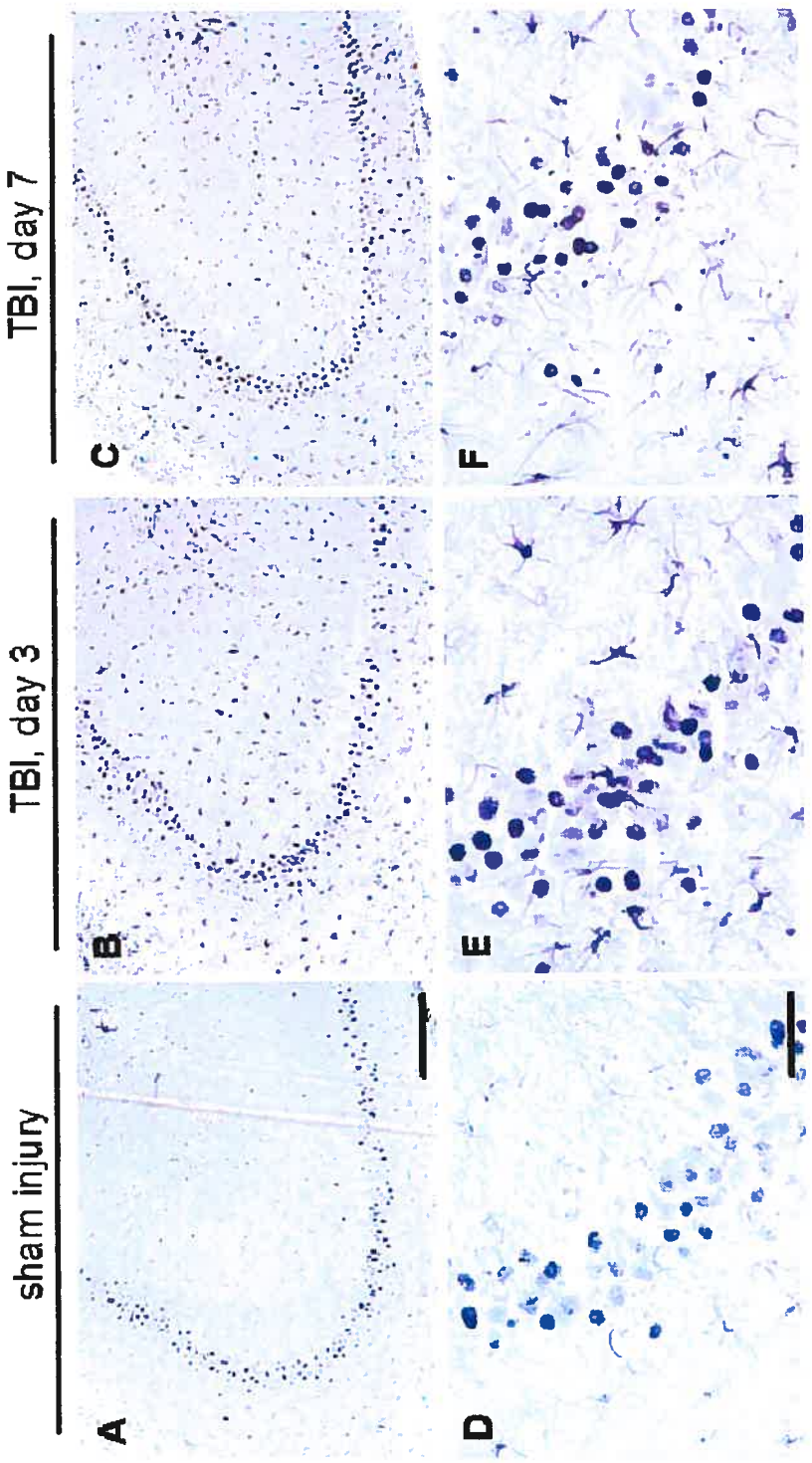
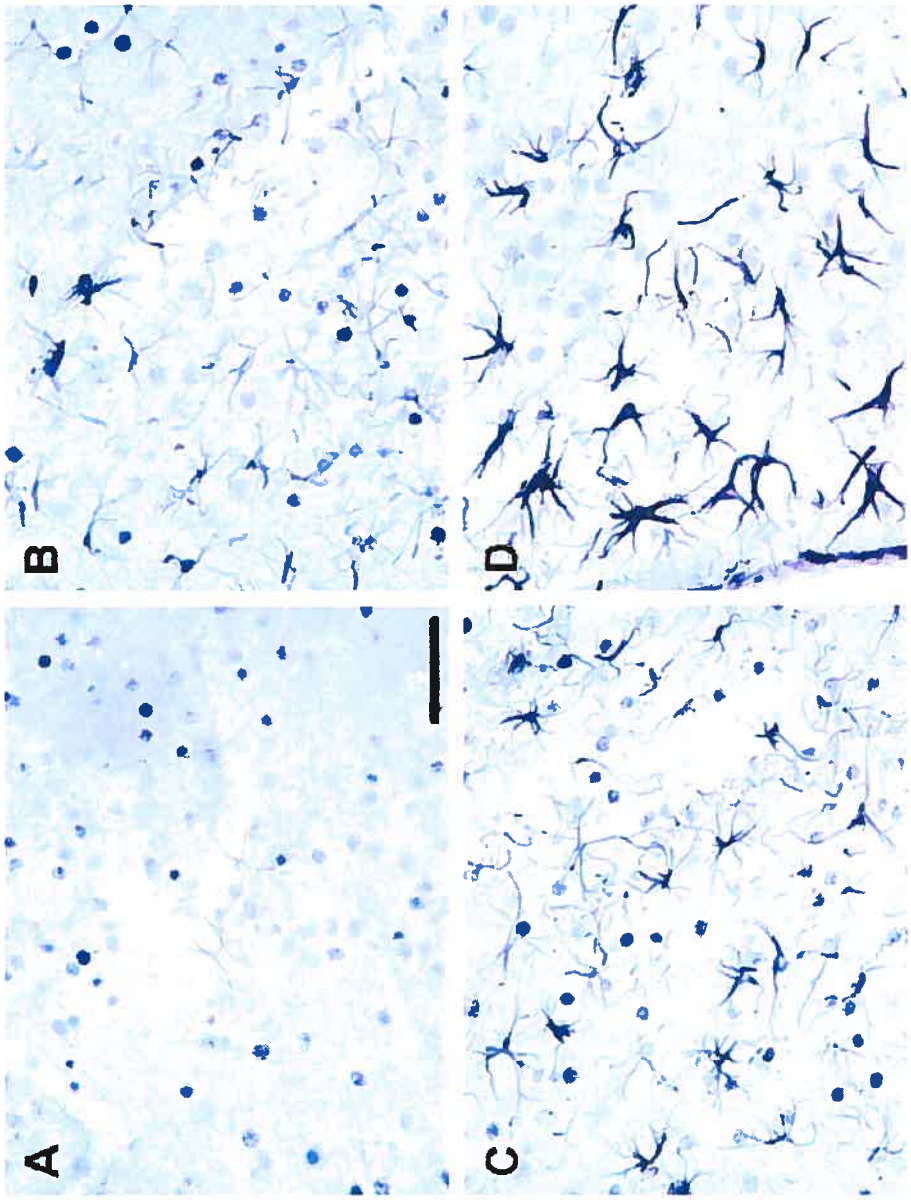

sham injury
TBI, day 3
TBI, day 7
A
B
C
D
E
F

Figure 4.

EAAT4 immunohistochemistry in cerebral cortex following sham (A), day 3 (B) or day 7 after TBI (C, D). Many astrocytes were stained positive for EAAT4 after the injury especially near the traumatic core (B, C) and glial limitans (D). Note peri-vascular localization of EAAT4 immunopositive cells. Bar; 50 μm.


B
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CHAPTER 3

DISCUSSION

3. General Discussion

This section aims to examine the relevance and significance of results obtained from the studies undertaken. Some unpublished data will also be discussed and, in conjunction with the data presented in the articles, will be used to draw a general conclusion of the thesis.

3.1 Contribution of glutamate transporter changes following TBI

3.1.1 Discussion on key points of Article 1: Early loss of protein expression of GLT-1v in the injured cerebral cortex following FPI

To determine the potential role of high-affinity sodium-dependent glutamate transporters in the early pathophysiology of experimental trauma, semi-quantitation of immunoblot bands and immunohistochemical staining of these proteins were employed to study the rat brains following acute FPI (6 and 24 hrs) or sham injury utilizing polyclonal antibodies specific for each transporter protein. From such experiments, the following results were obtained; 1. Glutamate transporter GLT-1α, GLAST, and EAAC1 showed no change following insult in the injured cerebral cortex, 2. Level of GLT-1v, the C-terminal splice variant of GLT-1α, decreased progressively in the injured cerebral cortex, 3. Injured hippocampus showed an increased relative immmunoreactivity of GLT-1α, but not GLAST and EAAC1, 4. Injured thalamus showed no change in the relative immunoreactivity for GLT-1α, GLAST, and EAAC1, 5. Both injured hippocampus and thalamus showed transient decrease in the relative immunoreactivity in GLT-1v at 6h following injury. These alterations in the protein levels of glutamate transporters were brain region specific, and suggest a differential but unique contribution of the transporters in these three regions of the brain in the development of neurodegeneration following FPI. As loss in the protein content of glutamate transporters influences the glutamate clearance capacity of that region, an increase in extracellular glutamate concentration is possible, hence resulting in a potential for secondary excitotoxic injury via neurotoxic action of glutamate. In

addition, the current finding showing the loss of GLT-1v, a C-terminal splice variant of major astrocytic brain glutamate transporter GLT-1α, represents a unique opportunity whereby the functional role of two splice variants can be studied separately from each other.

3.1.1.1 Mechanism(s) involved in loss of GLT-1v protein following FPI

Although the decrease in the GLT-1v protein level was not followed up by an investigation of change in the messenger RNA level following trauma, it is apparent that the loss of GLT-1v protein is not likely due to a loss in the messenger RNA as the protein turnover rate (degradation-*de novo* synthesis) of glutamate transporter GLT-1 protein is typically at least 48 hrs (Zeleania and Robinson, 2000) and the earliest time that loss of GLT-1 transporter protein has been detected is 2 hrs following trauma (Rao et al., 1998). It is thus likely that a selective degradation via proteolysis (or other undefined active processes) accounts for this downregulation that occurs after trauma.

According to Beckstrøm et al (1999), the C-terminal amino acid sequences of GLT-1α are more vulnerable to protein degradation-related loss of immunoreactivity, and the loss of immunoreactivity does not necessarily correlate to loss of [^{3}H]D-aspartate 'binding' nor the glutamate uptake activity from reconstituted liposomal preparations. In this respect, the selective loss of GLT-1v reported in the present study is interesting at many levels. First, the loss of GLT-1v, and not GLT-1α, questions whether this loss is due to ubiquitous degradation of c-termini of glutamate transporters following FPI. Rather, it seems that some other mechanism(s) that leads to a selective splice variant specific degradation is acting.

In primary cultures of non-differentiated astrocytes, the PKC-dependent phosphorylation mechanism could result in the loss of immunoreactivity of GLAST, but not the decrease of protein and function itself, suggesting a regulatory mechanism of GLAST whereby an apparent uncoupling of transporter immuno-content versus transport activity occurs (Susarla et al., 2004). If PKC-dependent phosphorylation is considered, it is important to keep in mind that the consensus PKC-phosphorylation sites are conserved between GLT-1v and GLT-1α splice variants, unless there are

unidentified phosphorylation site(s) within the 12 amino acids unique to GLT-1v. Since it shares high homology with GLT-1α, the selective downregulation will not occur if the mechanism of loss of immunoreactivity (downregulation) is due to PKC-dependent phosphorylation on the consensus phosphorylation sites alone. However, in their study, Susarla et al (2004) suggests the downregulation of GLAST to be due possibly to phosphorylation on the GLAST protein sites that are not consensus PKC-phosphorylation sites, as GLAST is known to be phosphorylated in other regions as well (Conradt and Stoffel, 1997). So far, the phosphorylation-dependent (and activity-independent) loss of immunoreactivity like that of GLAST (Susarla et al., 2004) has not yet been reported for GLT-1. In addition, since there was no detectable change in the GLAST level following trauma, it is unlikely that such a phenomenon is at play in the traumatized brain, but rather is likely to be confined to *in vitro* cell culture setting (primary astrocytic culture).

3.1.1.2. Potential contribution of the loss of GLT-1v protein in the pathophysiology of trauma

The relatively narrow appearance of GLT-1v band on the gel is suggestive of a low amount of this protein present in the given region. No significant downregulation of GLT-1 was observed following FPI when an N-terminal specific antibody was employed (unpublished observation), nor GLT-1α using a specific C-terminal antibody (this article, Figure 2). If the immunocontent of GLT-1v were to be of significance in the whole GLT immunocontent, one might expect that a decrease of 80% in relative immunoblot intensity of GLT-1v seen in the cerebral cortex 24 hr after FPI should have been detected, albeit relatively less pronounced, when N-terminal pan-GLT antibody was employed. The lack of this detection suggests considerably lower amount of GLT-1v present in the cerebral cortex in relation to GLT-1α. In support of this view, a significantly high amount of protein samples are loaded on the gel for immunoblotting of GLT-1v (50 μg/well), whereas only one fifth of that amount was used for GLT-1α, and yet the difference in the immunoblot band intensity between the two splice variants was quite huge, with GLT-1α band intensity much

more pronounced compared to that of GLT-1v. This view is in conflict with Chen et al (2002) whereby the protein level of GLT-1α and that GLT-1v were shown to be comparable in many regions of the brain, as well as in primary astrocytes after dibutyryl cyclic AMP treatment by western blot. However, in the successive publication, Chen et al (2004) showed concern regarding the authenticity of their paper in 2002 for the specificity of GLT-1α and GLT-1v antibodies, and further showed that the messenger RNA distribution of GLT-1α to be the predominant isoform expressed in the brain (Berger et al., 2005). Despite these findings, the contribution of GLT-1v in removal of interstitial glutamate in respect to its localization and relative expression level in different regions of brain remains to be elucidated.

The formation of multimer bands for GLT-1v suggests that this protein has the characteristics of a glutamate transporter (Haugeto et al., 1996). When brain sections were examined by immunohistochemistry, the GLT-1v antibody revealed astrocytic labeling pattern in the cerebral cortex and hippocampus approximating, yet unique from that of GLT-1α, but also further away from synapses, staining astrocytic somata rather than processes, in line with previous publications (Reye et al., 2002; Sullivan et al., 2004). Although results from the testing of antibody via immunochemical procedures are in line with glial GLT-1 in the literature, the unequivocal test to show the authenticity of antibody binding will require the use of a knockout. The polyclonal antibodies to GLT-1v and GLT-1α (not affinity purified, but supplied in crude serum) that were employed in our study were provided by Dr. David V Pow, but there have been no knockout studies to verify the authenticity of labeling. It is questionable that generation of selective GLT-1v-null mice can be established; however, for the purpose of validating antibody specificity, one might consider testing the authenticity of antibody on preexisting GLT-1 null mice (Tanaka et al., 1997) in the future since both GLT variants will be knocked out.

As indicated in the Discussion section of the first article, it is likely that the unique C-terminal PDZ-binding sequence provides a clue to the exact role of the GLT-1v splice variant in the brain. The recent finding that the PDZ sequences in the GLT-1v interact with PICK-1 (Chen and Rosenberg, 2002), and that there may be an

interaction of PICK-1 and the glutamate metabotropic receptors, suggest an interesting avenue of research for future studies.

3.2. Contribution of complexin I and II changes following TBI

3.2.1 Discussion on key points of Article 2: Early, transient increase of complexin I and II in the cerebral cortex following traumatic brain injury is attenuated by *N*-acetylcysteine

If the main function of complexins I and II in SNARE-mediated synaptic exocytosis is the stabilization of SNARE complexes in a tight conformation thus maintaining a highly Ca^{2+} sensitive pool of ready releasable vesicles (Reim et al., 2001; Chen et al., 2002), then the transient increase in the complexins level in the injured cerebral cortex following FPI would favor the likelihood of increased exocytosis and subsequent rise in extracellular glutamate concentration.

Interestingly, the acute, transient increase of complexin I and II protein level after injury (Article 2, Figure 2) is independent of messenger RNA levels, when RT-PCR was performed (unpublished findings). At 6 and 24 hr following injury, complexin I messenger RNA levels showed no change, whereas that of complexin II decreased at 6 hrs and remained depressed up to 24 hrs following FPI. It is thus likely that there exists a dysregulation between the messenger RNA level and that of protein level, and that this dysregulation is associated with oxidative stress-related events, since antioxidant treatment inhibits the transient increase in both complexins protein levels following injury (Article 2, Figure 5).

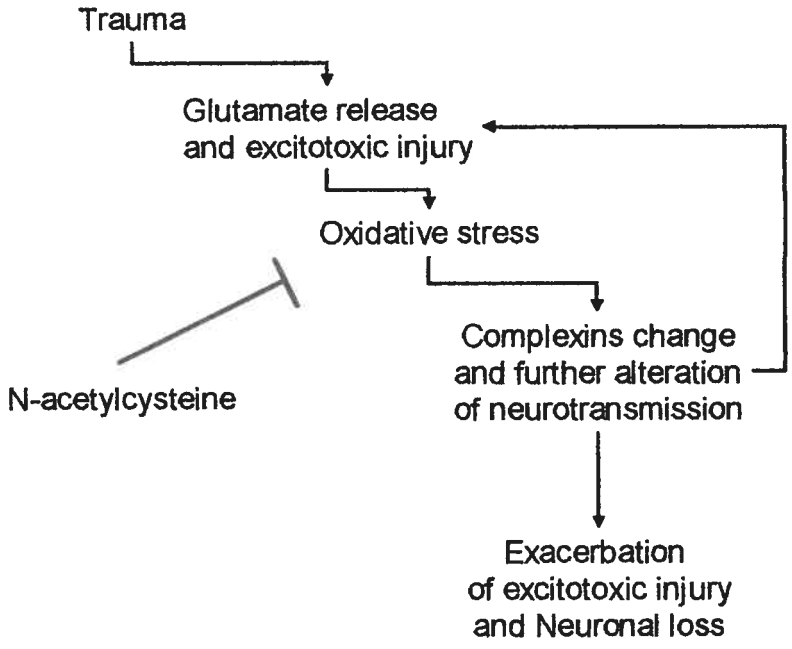

Figure 3.1. *Simplified diagram demonstrating the progression of neuronal loss and excitotoxic injury and their association with oxidative stress-related alteration of complexins level. Note that the action of N-acetylcysteine on reducing oxidative stress is upstream of the alteration of complexins and exacerbation of excitotoxic damage.*

Qualitative assessment of oxidative damage has shown increased 3-Nitrotyrosine (NT)-positive neurons in the injured cortex following FPI that displayed similar localization with complexin I and II (Article 2, Figure 3). It seems likely, therefore, that oxidative damage in neurons following trauma may be responsible for the altered level of complexins detected in the injured cortex. Treatment with *N*-acetylcysteine significantly reduced the number of NT-positive neurons, and completely abolished the transient rise in complexins in the injured cortex area, suggesting the alteration of complexins to be downstream of the oxidative stress-related event (Figure 3.1).

3.2.2. Localization of complexins and its role in the brain

Localization study in cerebral cortex and hippocampus showed complexin I to be found mostly in axosomatic synapses whereas complexin II in axospinal and axodendritic, suggestive of inhibitory and excitatory synapses, respectively (Yamada et al., 1999). However, this is not the case for all brain regions, since in mouse striatum complexin II messenger RNA is expressed by inhibitory output neurons (Freeman and Morton, 2004). This, with the lack of knowledge of the exact function of these proteins, makes it difficult to interpret the present results. Moreover, a new report of other forms of complexins III and IV in the brain and at the Ribbon synapse of retina represents an additional level of complexity to the story (Reim et al., 2005).

Both complexin I -/- mice (Glynn et al., 2005) and complexin II -/- (Glynn et al., 2003) mice survive whereas the double mutant of complexin I and II -/- die at birth (Reim et al., 2001). Complexin I -/- mice show severe ataxia, tremor and dystonia whereas complexin II -/- mice show abnormalities in exploration and social behavior, subtle progressive deficits in motor coordination, learning and reversal learning (Glynn et al., 2003; 2005). In experimental Wernicke's encephalopathy, there is a downregulation of both complexin I and II proteins in the thalamic area, and this downregulation is rescued by *N*-acetylcysteine treatment (Hazell and Wang, 2005), suggesting the depletion is mainly due to oxidative stress-related events. Since complexin I is highly expressed in thalamus, and considering the functional significance of the thalamus in relaying information from the cerebral cortex to cerebellum and basal ganglia thus contributing to higher-order control, loss of complexin I protein in the thalamus of experimental Wernicke's encephalopathy may also contribute to the development of ataxia and loss of righting reflexes. In the injured cerebral cortex of FPI rats, the transient rise in complexin levels may reflect increased sensitivity to Ca^{2+}-dependent release of neurotransmitters, while the loss in the complexin I level in the injured cerebral cortex and hippocampus at later time points following trauma may predispose towards an increased likelihood of post-traumatic seizure activity.

3.3. Involvement of oxidative stress in glutamate transporter regulation following FPI

3.3.1. Lack of effect of *N*-acetylcysteine on the loss of GLT-1v early following TBI

Treatment of rats with *N*-acetylcysteine post-injury reduced the early transient increase in protein content of complexins I and II in the injured cerebral cortex, as well as decreased the injured volume and neuronal loss of the region. However, the same treatment failed to block the decreased GLT-1v level in the injured cerebral cortex detected following FPI (Figure 3.2), despite the fact that NAC treatment reduced the amount of detectable 3-Nitrotyrosine immunoreactivity. This suggests that the loss of GLT-1v immunoreactivity is an independent event from that of oxidative stress and runs parallel to the oxidative stress-induced alteration of neurotransmitter release/neuronal injury, reflecting a more complex pathophysiology.

The early detection of increase in immunoreactivity of 3-nitrotyrosine (as early as 6 hr) suggests that there may be nitrosative damage following injury, and potential production of $ONOO^-$ that might contribute to the inactivation of glutamate transport function (Trotti et al., 1996), in addition to the proposed alterations in complexin levels (Article 2). Thus, the reduction in the injury volume by NAC treatment following FPI may be due to the reduction of $ONOO^-$ production and reduction of transporter inactivation that occurs early in the injury process. This represents a valid and interesting avenue for research following that presented in this work and merits further consideration.

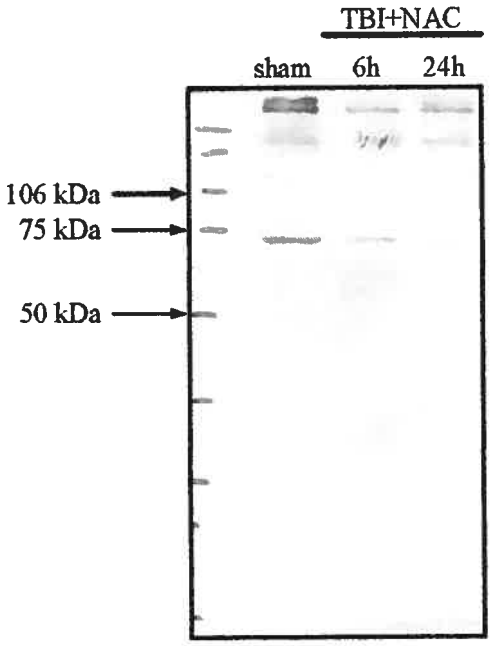

Figure 3.2. *Representative bands for GLT-1v immunoblots. Sham injury (sham), 6 h TBI with NAC (6h TBI+NAC), and 24 h TBI with NAC (24h TBI+NAC) in the cerebral cortex of the rat following FPI of moderate severity.*

3.3.2. Oxidative cross-linking of glutamate transporters at later stages of TBI suggests involvement of oxidative damage-related dysfunction of glutamate transporter

Though there is no significant change in the protein levels of GLT-1α and GLAST in the injured cerebral cortex of FPI rats, there is, however, an increased injury-dependent cross-linking of glutamate transporters. When protein samples were prepared according to our protocol, a certain percentage of glutamate transporter oligomer bands appear that is irreversibly cross-linked by reducing agent DTT. When run under non-reducing gel and blotted against GLT-1α– and GLAST-specific antibodies, protein samples of injured cerebral cortex at days 3 and 7 following injury

showed an additional increase in the band intensity of oligomers at the expense of monomer band (Figure 3.3). Interestingly, this was fully reversible under reducing conditions (Figure 3.4), suggesting a likelihood of severe tissue oxidation-induced SDS-independent sulfhydryl cross-linking of glutamate transporters (Haugeto et al., 1996). This was an injury specific event that only occurred in the trauma-affected ipsilateral cerebral cortex of day 3 and 7 TBI rats prepared under same conditions (and not before), when cross-compared with the contralateral brain of the same animal on the gel.

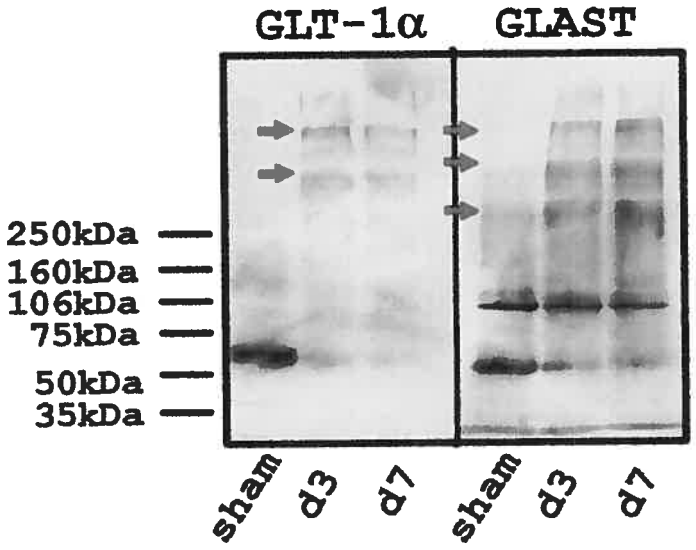

Figure 3.3. *Enhanced multimer formation of GLT-1α and GLAST glutamate transporter proteins at days 3 and 7 following trauma in the injured cerebral cortex. Samples were loaded in a 3-8% gradient gel and run under reducing conditions. Arrows indicate the presence of multimer forms of the transporters.*

At present, it is unclear as to whether the oligomerization promoted at these time points represents solely cross-linking of glutamate transporters to themselves, or the involvement of other protein(s) as well and thus a non-specific protein aggregate

formation. When beta-actin was immunoprecipitated from the injured cerebral cortex with monoclonal beta-actin primary antibody at day 3 following FPI, the resulting immunoprecipitation product was not immunoreactive with glutamate transporters GLT-1α or GLAST primary antibody, suggesting the potential involvement of beta-actin molecules in the transporter oligomerization is unlikely. Further studies are required to determine whether other proteins are involved.

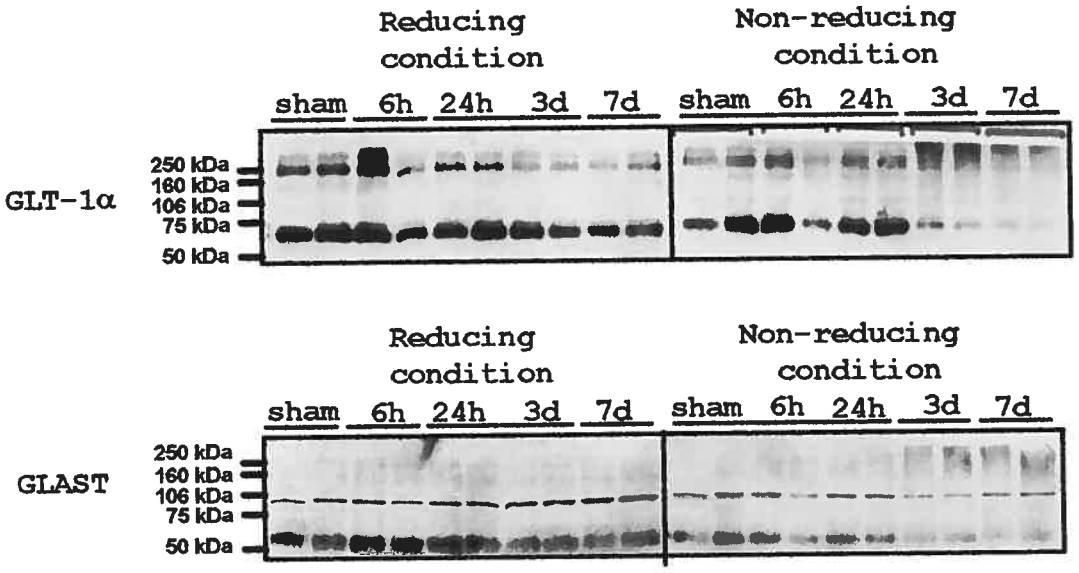

Figure 3.4. *Reversible oxidative cross-linking of GLT-1α and GLAST occur at days 3 and 7 after FPI, but not at earlier time points. Samples were run under reducing conditions (left) or non-reducing conditions (right) and immunoblotted with GLT-1α or GLAST antibodies.*

The rat glutamate transporters EAAC1, GLT-1, and GLAST carry 6, 9 and 3 cysteine residues in their sequences respectively. An *in vitro* study suggests that the oxidation and cysteine sulfhydryl cross-linking of glutamate transporters GLT1, GLAST and EAAC1 significantly reduces glutamate uptake and uptake associated current, and this was reversible by application of reducing agent DTT (Trotti et al.,

1997a, 1997b, and reviewed in 1998). Given the extent of oxidative cross-linking seen in the injured cerebral cortex, it is likely that the glutamate transport activity of GLT-1α and GLAST is significantly reduced despite the lack of detection of decreased protein levels at these time points. Based on the knowledge that the extensive oxidative cross-linking may result in the proteolytic degradation process, it may be possible that a slow degradation of glutamate transporters develops in this brain region at much later time points. This interesting event, in conjunction with the early detection of protein nitrotyrosine suggests an involvement of oxidative stress-related inactivation in the function of glutamate transporters with no direct connection to losses in glutamate transporter protein levels following FPI.

3.4. Discussion on key points of Article 3; EAAT4 protein increases in the hippocampus following FPI

Although the loss of glutamate transporter GLT-1α and GLAST reported in CCII (Rao et al., 1998) was not seen at early time points following FPI, it is possible that the loss in glutamate transporter levels could occur at later times, this being part of the rationale for extending the time points investigated following FPI. When glutamate transporter levels were examined at later time points (3 days and 7 days) following FPI, there was little or no difference in the immunoblot intensity of GLT-1α, GLAST and EAAC1 in the injured cerebral cortex, whereas GLT-1v levels were low and sustained up to 168 hrs in the injured cerebral cortex (unpublished findings). Thus, it became rather clear that in this model of experimental trauma, there was no downregulation of GLT-1α and GLAST, but only GLT-1v, unlike the case of CCII (Rao et al., 1998). However, when another sodium-dependent glutamate transporter EAAT4 was examined, an increased level in the injured brain at these later time points was detected.

EAAT4 protein levels studied by semi-quantitative immunoblotting revealed that, in the injured hippocampus, there was a modest increase of 20 and 25 % following 72 hrs and 168 hrs trauma, respectively (Article 3). A similar increase was not seen in other regions such as injured cerebral cortex and thalamus by this

method, probably due to very low amounts of EAAT4 in the forebrain (Dehnes et al., 1998). Surprisingly, when studied via immunohistochemistry, this increase was primarily astrocytic in nature. When normal brain sections were re-examined by EAAT4 immunohistochemistry, astrocytic staining was detected, albeit low, throughout white matter tracts of the forebrain and also in the granule cell layer of cerebellum (Article 3, Figure 1) as well as in the brainstem area. Very little or no astrocytic staining was detected in the grey matter of forebrain region.

The injured cerebral cortex showed a heterogeneous induction of EAAT4, where some areas showed increase and others no increase in the affected hemisphere. In the hippocampus 3 and 7 days after trauma, however, the EAAT4 positive immunoreactivity was more or less homogeneous. In the injured thalamus area, EAAT4 labeling was even less than that of cerebral cortex. This regional disparity in increased EAAT4 levels, along with the low basal level of this protein may account for the lack of detection of significant increase in these two areas, when examined by immunoblotting. At the earlier time points such as 6 or 24 hr following trauma, EAAT4 labeling was no different than that of sham brain, suggesting this increase is a late event.

3.4.1. Specificity of immunolabeling

Several important matters should be considered regarding this study. First, the literature indicates the EAAT4 transporter is expressed exclusively in neurons, mostly in cell body and dendrites of Purkinje cells in the molecular layer of cerebellum in normal rat brain (Dehnes et al., 1998). Using EAAT4 antibody, a small fraction was immunoprecipitable from the forebrain region, whereas immunohistochemistry failed to demonstrate any detectable labeling in this area (Dehnes et al., 1998). However, there are some references supporting an astrocytic EAAT4 localization in the retina, spinal cord and forebrain of rat and mice (Ward et al., 2004; Hu et al., 2003), as well as in hippocampus (Furuta et al., 1997). It remains questionable as to whether this EAAT4 labeling represents labeling of the EAAT4 glutamate transporter, since none of the reports included a knockout study to support their finding, including that of

Dehnes et al (1998).

The second issue is the presence of nuclear staining in the immunohistochemical sections displayed in this study. EAAT4 is a glycosylated membrane protein and hence the presence of nuclear staining can pose a serious doubt about the reliability of our findings. In addition, observation of nuclear staining is not uncommon and likely to represent artifactual result when high phosphate buffer is used; this nuclear staining was later completely eliminated by increasing the blocking condition and replacing the PBS buffer with TBS (Figure 3.5) in the immunohistochemical condition. This method was preferred as the antibody performance is at its best in the condition it is affinity-purified (TBS) (Lehre and Danbolt, 1995). Despite the significant reduction of nuclear staining, the astrocytic staining persisted (or became even more pronounced), supporting the notion of low basal astrocytic EAAT4 expression throughout the white matter of forebrain region (Hu et al., 2003).

Since the astrocytic labeling of EAAT4 resembled that of GFAP, it was possible that the staining pattern represents a non-specific cross-reactivity with GFAP, based on the similarity of cytoplasmic/cytoskeletal labeling. This is an interesting possibility, since the antibody used for present study is not a monoclonal one, and the extent of purity and homogeneity of the antibody is not well defined. Although the answer to the antibody specificity remains unclear at this time, it is an interesting point as to why some astrocytes are stained positive for EAAT4 and that not all GFAP positive astrocytes are stained positive for EAAT4 in the region. In the cerebral cortex and hippocampus, EAAT4 labeled astrocytes are scarce or not present in the grey matter, but instead are concentrated in the corpus callosum and internal capsule. In addition, EAAT4-labeled astrocytes resemble fibrous astrocytes, and it is difficult to tell whether the labeling is cytoskeletal or membranal at the light microscope level (Figure 3.5). This puts the notion of a cross-reactivity theory in doubt, though it does not completely rule out a possible cross-reactivity that is of non-GFAP but selectively astrocytic in nature. The epitope sequence of EAAT4 antibody used in this study is same as that used in Dehnes et al (1998), where the antigenic sequence corresponds to the C-terminal sequences of rat EAAT4. The antibody that gave rise to mice spinal

cord and rat retinal astrocytes used by Hu et al (2003) and Ward et al (2004) study came from a commercially available source (Alpha Diagnostic, San Antonio, Texas, USA), and the C-terminal targeting antigenic sequence differs from that used in this study. Thus, at least two different antibodies have been used to support the astrocytic labeling in the rat and mice brain. If the non-specific cross-reactivity appears specific by more than one observer, it is likely to be specific labeling unless proven otherwise.

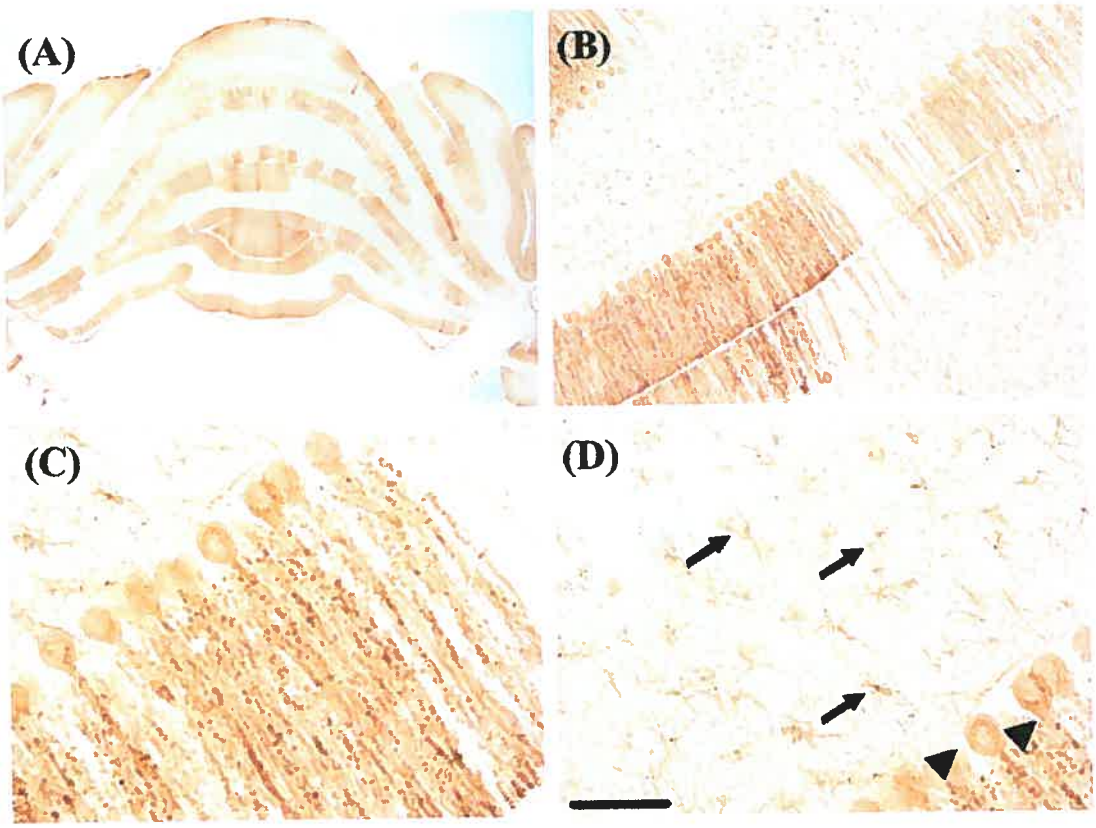

Figure 3.5. *EAAT4 immunohistochemistry in coronal section of rat cerebellum 3 days following FPI. Bar; 0.2 mm for B, and 50 μm for C and D. In D). Arrows point to astrocytic labeling in the granule layer of cerebellum, whereas arrow heads point toward Purkinje cells. Method modified from Lehre and Danbolt (1998).*

When examined under other laboratory settings (Dehnes et al., 1998), there was no detectable EAAT4 immunoreactivity at all in the forebrain region using 0.47

μg/ml antibody. This is in direct conflict with the very same study showing immunoprecipitable amounts of EAAT4 present in the forebrain region, as well as our western blot study showing trace amounts of the protein in the cerebral cortex and hippocampus area. It is very difficult to embrace our labeling as being a non-specific cross-reactivity because; 1. The relative staining intensity is in alignment with the immunoblot data when compared between samples from forebrain regions and that of cerebellum; 2. At least 3 different investigators (laboratories) utilizing at least 2 different antibodies prepared with different epitope sequences have shown similar results.

Following trauma, reactive gliosis occurs as early as 24 hrs whereas the increase in EAAT4 positive astrocytic labeling only appears at later time points (72 and 168 hrs). The EAAT4 immunoblot band showed both monomer and dimer constituents in the hippocampus that are at the appropriate molecular weight (64 kDa and 130 kDa, respectively), and do not show bands of other molecular weight. Overall, there are several observations that presently support the likelihood of astrocytic EAAT4 increase in the forebrain region in response to FPI. This astrocytic labeling should eventually be verified using EAAT4 knockout mice.

CHAPTER 4.

CONCLUSIONS

4. Conclusions

Clearance of glutamate in the brain is performed by a family of sodium-dependent glutamate transporters. Because the termination of glutamatergic synaptic signals is performed mainly by uptake of glutamate by these transporters, it is crucial to maintain normal levels of these proteins for proper clearance of glutamate. A compromise of this situation can produce excitotoxicity via a rise in the interstitial glutamate concentration.

In this thesis, it was initially considered that protein levels of GLAST and GLT-1 decrease following experimental trauma which contributes to the rise in brain interstitial glutamate concentration observed in this model. Instead, GLT-1v, a splice variant of the glutamate transporter GLT-1α, decreases progressively in the injured cerebral cortex acutely (6 and 24 hrs) following FPI that correlates positively with the neuronal loss seen in the same area. At the same time, other glutamate transporters showed little or no change. In addition, there was little or no indication of changes in the levels of other glutamate transporters in the injured cerebral cortex at later time points (days 3 and 7) following FPI, while GLT-1v is sustained at low levels up to 7 days following trauma. On the other hand, EAAT4 protein levels were increased at these time points following FPI throughout the injured hemisphere and appeared to be also astrocytic in its localization. This rise in EAAT4 levels at later time points may therefore represent a compensatory mechanism to counteract the decrease in GLT-1v levels detected in the injured brain.

Also considered was the possibility that alterations in complexin levels may contribute to the increase in the extracellular glutamate levels known to occur following brain trauma. Experimentally, it was shown that an early transient increase in protein content of complexin II occurred following brain injury that appeared to be due to oxidative stress. This upregulation in complexin II may be partly responsible for the acute increase in interstitial glutamate concentration seen in this model. The results also indicate that oxidative stress may exacerbate brain injury in this model, as *N*-acetylcysteine reduced injury volume and neuronal loss.

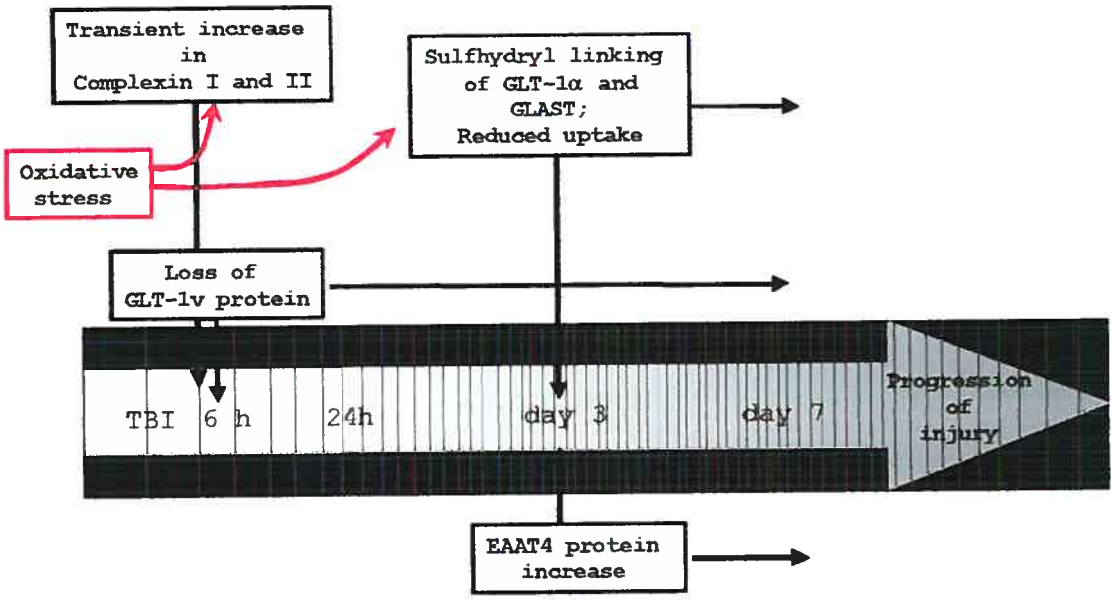

Figure 4.1. *Schematic diagram summarizing events following FPI.*

At days 3 and 7 following trauma, glutamate transporters in the injured cerebral cortex displayed evidence of oligomerization, migrating at higher molecular weights representative of multimers. This finding also supports the notion that oxidative damage affects the post-traumatic brain, in this case causing transporter proteins to form sulfhydryl bonds. Such a condition has previously been reported to reduce glutamate transport activity *in vitro* (Trotti et al, 1997a).

Overall, these results provide evidence of excitotoxic damage in which oxidative stress influences both glutamate transporter function and regulation of complexin levels. Oxidative stress gives rise to a transient increase in complexin I and II levels in the injured cortex, which may result in more neurotransmitter release. Glutamate transporter GLT-1v protein levels decrease in a parallel but independent fashion, contributing to the excitotoxic event in the injured cerebral cortex. At later times, however, oxidative stress facilitates sulfhydryl cross-linking of glutamate transporters GLT-1α and GLAST that can further exacerbate the excitotoxic injury.

These results are summarized in Figure 4.1. Thus, it appears that impaired glutamate uptake due to altered levels of glutamate transporters, in conjunction with changes in neurotransmitter release due to altered complexin levels, together play an important role in the pathophysiology of TBI.

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