# Chapter 6
# Role of Astrocytes in Central Nervous System Trauma

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**Abstract** In the blink of an eye, the life of a person who sustains a traumatic brain injury (TBI) or traumatic spinal cord injury (SCI) often changes forever. This chapter discusses the epidemiology of SCI and TBI followed by an overview of the key elements known to contribute to the pathophysiology. Next, astrocytic physiology thought to be involved in the acute post-injury phase is discussed, with particular focus on the mechanisms of protection.

**Keywords** Central · Nervous system · Astrocytes · TBI · Traumatic brain injury · SCI · Traumatic spinal cord injury · Glutamate · Kir4.1 · EAAT · Excitotoxicity · Inflammation · Plasma

## 6.1 Epidemiology of Traumatic Central Nervous System Injury

Traumatic spinal cord injury (SCI) is a major health concern in the United States (U.S.) and world not only due to the number of persons with SCI, but also due to the cost of long-term care of the SCI patient (Jackson et al. 2004). Estimates from 2013 of the incidence of SCI in the U.S. report that there were approximately 40 cases per million population, not including persons who die at the scene (National Spinal cord Injury Statistical Center 2013). This constitutes nearly 12,000 new SCIs occurring each year in the U.S. with most newly injured persons being in the 16 to 30 year old age group. Also, most persons (80.7 %) who sustain an SCI are males (National Spinal cord Injury Statistical Center 2013). The main etiology for SCI is motor vehicle crashes (36.5 %), followed by falls (28.5 %), and acts of violence (14.3 %), primarily gunshot wounds. Although often high-profile, SCIs caused by sports represent only 9.2 % of the total SCI annual incidence. A person who sustains an injury at one of the eight cervical segments of the spinal cord suffers from tetraplegia. An injury

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in the thoracic, lumbar, or sacral regions of the spinal cord results in paraplegia. The majority of persons with an SCI sustained tetraplegia (52.2%) (National Spinal cord Injury Statistical Center 2013; Jackson et al. 2004).

The cost of SCI is staggering on an individual, community, and national level. It is important to note that the annual average health care, living expenses, and estimated lifetime costs that are directly attributable to the injury vary greatly by severity of injury and do not include indirect costs such as lost wages and productivity, which average US$ 70,575 per year in 2013 (National Spinal Cord Injury Statistical Center 2013). The average yearly expenses for the first year post-SCI in the USA is US$ 1,044,197 for high tetraplegia, US$ 754,524 for low tetraplegia, and US$ 508,904 for paraplegia. The estimated life time cost (not including indirect costs) for a patient injured at age 25 is between US$ 3.3–4.6 million for tetraplegia and US$ 1.5–2.2 million for paraplegia (National Spinal cord Injury Statistical Center 2013). SCI significantly lowers the average life expectancy as well. For example, a person who sustains paraplegia at 20 years of age who survives at least 1 year post-SCI has an average reduction in life expectancy of up to 13.9 years as compared to a person without SCI. For a person with tetraplegia, the average reduction in life expectancy is 18.6 (National Spinal cord Injury Statistical Center 2013).

Traumatic brain injury (TBI) also constitutes a world-wide concern to human health. In the U.S., the annual incidence of TBI is estimated at 1.7 million, and TBI is a contributing factor to 30.5% of all injury-related deaths. Additionally, the yearly economic burden (includes direct and indirect costs) was estimated in 2000 as US$ 76.5 billion in the US, which does not include the social and emotional toll on patients, families, and the community (National Center for Injury Prevention and Control 2013). The age groups most likely to sustain a TBI are children aged 0 to 4, older adolescents aged 15 to 19, and adults aged 65 and older (National Center for Injury Prevention and Control 2013). When considering TBI in only persons 16 years or older, 48% of TBIs occur in persons age 16–35 and persons in the 36–56 year old categories constitute 40% of TBIs (National Data and Statistical Center 2013). As with SCI, injury occurs in males (74%) more than females (26%) at any age (National Data and Statistical Center 2013). Recent evaluation of the etiology of TBI in adults indicates that 53% of TBIs were the result of motor vehicle crashes, followed by 24% caused by falls, and 13% from violence. Evaluation of the blood alcohol (BAL) level at time of emergency department admission shows that 54% of persons who sustained a TBI were negative and 43% had a BAL of greater than 10 mg/dl (National Data and Statistical Center 2013) (note that 80 mg/dl is considered positive for driving under the influence in most states). Duration of loss of consciousness after TBI is highly correlated with injury severity and long-term outcome. The National Data and Statistical Center (NDSC) TBI model system data indicated that the average duration of loss of consciousness for that data set was 8.3 days. The average duration of post-traumatic amnesia is 24.3 days in the NDSC data. At 1 year post-TBI, 34% of persons who sustained a TBI exhibit moderate to severe disability and 36% exhibit mild or partial impairment. Data also show that a 1 year post-TBI, 30 % of persons who sustained a TBI show no disability. Also, 38% of persons who sustained a TBI require some level of supervision at

1 year post injury and 33 % at 2 years post-TBI (National Data and Statistical Center 2013).

With regard to injury severity, the NDSC TBI model system data indicates that 46 % of TBIs are severe, 16 % are moderate, and 39 % are mild. However, the U.S. Centers for Disease Control and Prevention (CDC) estimate that approximately 75 % of the 1.7 million brain injuries in the U.S. are mild TBI. The data source is one likely cause of this discrepancy as the NDSC TBI model system data is comprised from hospital emergency department records of persons 16 years of age or older at the time of injury. In contrast, the CDC data sources include all age groups with date obtained from the National Hospital Discharge Survey, the National Hospitals Ambulatory Medical care Survey and the National Vital Statistics Systems for the years of 2002 to 2006 (Faul et al. 2010).

In addition to TBI in the civilian populations, it is now being recognized that military TBI is an overarching concern. For example, evaluation of U.S. Army personnel deployed to Iraq and Afghanistan, including soldiers deployed in Operations Iraqi Freedom and Enduring Freedom (OIF/OEF), indicated that during the 10 year period from 1997–2006 over 110,000 military personnel experienced at least one TBI (Wojcik et al. 2010). Similarly, a RAND report suggested that 20 % of deployed service personnel suffered a TBI (Burnam et al. 2009). Terrio et al. (2009; Brenner et al. 2010) reported that 22.8 % of soldiers in a brigade combat team returning from Iraq had clinician-confirmed TBI and that the majority of these TBIs were defined as mild. They also reported that soldiers with confirmed TBI were significantly more likely to report post-deployment somatic and neuropsychiatric symptoms than those with no TBI, which suggest that the health consequences of even mild TBI (mTBI) can be enduring. Moreover, predominate cause of mTBI in military populations is exposure to blast (Hoge et al. 2008). Indeed from a broader perspective, over 63 % of casualties from October 1, 2001 through February 28, 2011 in OIF/OEF and Operation New Dawn were caused by detonation of an explosive device. Thus taken together, epidemiological data indicate that mTBI induced either by blast or direct impact is a significant occurrence affecting both military personnel and civilians, and that mTBI can have lasting effects on well-being.

Although an array of symptoms has been documented as occurring after blast-induced or impact-induced mTBI, evaluating and characterizing these neurological sequelae remains an area of active clinical research. Commonly described symptoms include retrograde and/or anterograde amnesia, learning impairments, reduction in executive function, reduced concentration, mood disturbances, disrupted sleep patterns, aggression, and anxiety (Hoge et al. 2008; Sayer et al. 2008). However, it remains an active debate as to the extent to which anatomical and biochemical alterations in the brain from blast exposure cause these impairments. Indeed, the question as to the mechanism(s) by which blast exposure induces lasting cognitive deficits (i.e. anatomical vs. psychological) has been actively debated clinically since the term “shell shock” was first coined during World War I and then raised again with the term “postconcussion syndrome” used in World War II (Jones et al. 2007). The clinical assessment of blast-induced mTBI is particularly complicated by the absence of an overt (detectable) focal lesion(s) which is combined with the high

comorbidity of psychiatric disorders such as post-traumatic stress disorder (Bryant 2001). Adding to this confusion is the idea that personnel in modern conflicts are exposed to blast at close proximity and with improved body armored protection which impacts the survivability as well as the physical properties of the blast wave (Cernak and Noble-Haeusslein 2010; Risling et al. 2011). Add the related problem of the impossibility of documenting blast characteristics such as blast wave propagation, secondary acceleration/deceleration, heating, and emitted gases on an individual basis and this makes the understanding of the relationship between biomechanical loading and the sequelae of mTBI extremely difficult to acutely assess in the clinical arena (Risling et al. 2011). Thus, several research groups (including ours) have developed animal models of blast-induced mTBI (Cernak and Noble-Haeusslein 2010) in order to systematically evaluate many of these complicated issues.

## 6.2 Overview of the Pathophysiology of Traumatic CNS Injury

The initiating event in a traumatic central nervous system (CNS) injury (TBI or SCI) is a mechanical insult and the resulting mechanical response at the cellular level, termed *primary injury*. Following this initial primary injury is a set of complex and interrelated biochemical *secondary injury* mechanisms which means that the array of deficits following a traumatic event is dependent on the mechanical response to the insult and complex secondary injury mechanisms. This concept has been extensively detailed and reviewed (see Bramlett et al. 1997; Bramlett and Dietrich 2002; Adelson et al. 2000; Hall and Wolf 1986; Hall 2003; Levin 1990; Popovich and Jones 2003; Povlishock and Katz 2005; Raghupathi 2004; Smith et al. 1994, 2003; Whalen et al. 1999). However, it is important to emphasize that the primary injury is the trigger for the secondary injury cascade and that differing biomechanical loading scenarios induce a different injury response (Schmitt et al. 2010).

For example, in TBI, the dynamic loading that is associated with contact and non-contact load differ and result in characteristically different brain pathologies. Specifically, contact loading caused by direct contact of the head to/from an object typically induces focal tissue damage oriented in the direction of the force vector. Moreover, rapid contact loading produces stress waves that propagate within the skull/brain that can lead to a pressure gradient which results in a positive pressure at the site of impact (coup) and negative pressure on the side opposite the impact (contrecoup). This pressure gradient gives rise to shear strain throughout the brain tissue. In non-contact scenarios, the head and brain are loaded by inertial forces, also termed acceleration/deceleration forces. Inertial loading can either be translational or rotational, with focal brain injury more associated with translational loading and diffuse brain injury with rotational loading. In reality, acceleration of the head also occurs in contact loading so the separation of these biomechanical forces is

somewhat academic as the mechanisms described for contact loading also occur in acceleration/deceleration brain injury. Thus, it is well-established that TBI caused by acceleration or a blow to the head (impact-induced TBI), results in isochoric deformation of the brain tissue and a significant biomechanics literature concludes that shear leading to stretch is the primary mechanical mechanism of damage in TBI (Ommaya et al. 2002; Zhang et al. 2010).

Although the primary injury is the triggering event in traumatic injury to the brain or spinal cord, the secondary injury mechanisms represent the area with the most complex pathobiology where in understanding the interactions between astrocytes and other brain cells could be critical to the development of therapeutic approaches. The initiating event in CNS injury is a physical insult and the resulting mechanical response at the cellular level (primary injury). Acute plasma membrane damage resulting from the primary injury can have detrimental effects on neuronal and astrocytic function, including disruption of ion homeostasis, synaptic transmission, and axonal transport (Pettus et al. 1994; LaPlaca et al. 1997; Stone et al. 2004; Shi and Whitebone 2006). Membrane damage may initially manifest as non-specific pores (Geddes et al. 2003; Farkas et al. 2006) and initiate secondary damage such as receptor dysfunction (Hardingham 2009) and phospholipid breakdown through free-radical production and inflammatory processes (Farooqui et al. 2007), leading to cell death (Whalen et al. 2008). Excitotoxicity is an acute secondary event stemming from depolarization and excess excitatory amino acid release, leading to further disruption of ion gradients, glutamate release, and intracellular calcium rise (Agrawal and Fehlings 1996; Arundine and Tymianski 2003; Sahuquillo et al. 2001). Energy deficits occur due to increased demand (Ahmed et al. 2000), causing mitochondrial dysfunction and a drop in ATP (Sullivan et al. 1998, 2004), hindering energy dependent ion pumps (Faden et al. 1987). Free radicals, including nitric oxide (Cherian et al. 2004) and reactive oxygen species (Hall et al. 2004) are liberated, damaging DNA, cytoskeleton, membranes, other proteins, and normal gene expression. Cell death can be either necrotic and/or apoptotic (Kovesdi et al. 2007; Raghupathi 2004) via caspase and calpain activation (Liu et al. 2006; Ringger et al. 2004). Inflammatory factors are also released from cells (Lenzlinger et al. 2001), including astrocytes (Israelsson et al. 2008; Laird et al. 2008; Suma et al. 2008) and resident microglia (Koshinaga et al. 2007), as well as infiltrating macrophages (Utagawa et al. 2008), resulting in exacerbation of pro-inflammatory cytokine production and more cell death.

There are several mechanisms in this pathological secondary injury cascade wherein astrocytes may be key therapeutic targets. As such, Table 6.1 summarizes several injury mechanisms that could potentially serve as therapeutic targets to break the progression from mechanical insult to cellular dysfunction and death. These target pathways are arranged into broad categories: (1) acute damage and excitotoxicity; (2) free radical damage and compromised energetics; and (3) inflammation which, although not exhaustive, cover a substantial portion of the injury cascade. In this chapter, we focus on the role of astrocytes in the excitotoxic phase of the secondary injury cascade (Table 6.1, Column 1).

**Table 6.1** Categories of secondary injury mechanisms for selection of potential therapeutic targets

| Excitotoxic | Energetic/free radical | Inflammatory |
|---|---|---|
| Mechanical damage | Decreased cell respiration/ induced ischemia | Activation of microglia → Production of pro-inflammatory cytokines |
| Membrane disruption | Decreased ATP production/ levels | Recruitment of macrophages → Production of pro-inflammatory cytokines |
| Increased $[Na^+]_i$, $[Ca^{2+}]_i$ | Lipid peroxidation/formation of free radicals/nitrosylation | Activation of astrocytes → Production of pro-inflammatory cytokines |
| Disregulation of astrocytic $K^+$ and membrane potential | Mitochondria damage and opening of mitochondrial permeability transition pore | Subsequent cell death and production of pro-inflammatory cytokines |
| Excessive glutamate release | Cytochrome C release | |
| Ionotropic and metabotropic glutamatereceptors activation | Increased $[Na^+]_i$, $[Ca^{2+}]_i$ | |
| Ionic disregulation activates ATP-dependent $Na^+$, $Ca^{2+}$ pumps | Altered gene expression | |
| Alteration of glutamate uptake | Activation of caspases, calpains and apoptotic mechanisms | |
| Sequestration of $Ca^{2+}$ into mitochondria | | |

## 6.3 Role of Astrocytic Glutamate Transporters in Excitotoxicity

The term "excitotoxicity" is credited to Olney in 1969 (Olney and Sharpe 1969; Olney 1969) with the seminal demonstration that injection of the primary excitatory neurotransmitter in the CNS, L-glutamate, into mouse or primate brain caused destruction of tissue. Since then, it has become well-established that primary injury in the brain or spinal cord rapidly leads to high extracellular glutamate (Faden et al. 1989a; Bullock et al. 1995; Choi 1992). In other words, the metabolic and cellular derangements in the secondary injury phase are largely initiated by massive and indiscriminant release of glutamate into the extracellular space (Katayama et al. 1990). Mechanisms for this glutamate release include mechanical disruption of the cell's plasma membrane, the subsequent disregulation of ionic gradients (i.e. sodium and potassium), and resulting indiscriminate electrical discharge (Katayama et al. 1990).Furthermore, the death of astrocytes, caspase mediated degradation of glutamate transporters, and reversal of sodium dependent transport have all been implicated in the pathology of glutamate release following TBI (Zhao et al. 2003; Floyd et al. 2005; Floyd and Lyeth 2007).

Glutamate extracellular concentration must be maintained at low levels by a family of plasma membrane sodium dependent excitatory amino acid transporters (EAATs), which are selectively expressed throughout the mammalian brain on both neurons and glia. EAATs 1 and 2 (in human, while GLAST and GLT-1 in rodents, respectively; here, used interchangeably) are expressed primarily in astrocytes while the remaining transporters reside on neurons (Shigeri et al. 2004). The astrocytic glutamate transporters are crucial for the proper maintenance of extracellular glutamate levels; antagonists for them can lead to toxic levels of glutamate and subsequent cell death similar to that exhibited in TBI (Shigeri et al. 2004). Of the astrocyte transporters, EAAT2 is responsible for approximately 90 % of the

clearance of glutamate from the extracellular space (Kanai and Hediger 2003). The function of this glutamate transporter is significantly reduced following TBI in rat cortical tissue, as illustrated by decreases in both mRNA levels and protein expression of EAAT2 following TBI (Rao et al. 2001). Other studies using TBI rodent models have reported comparable decreases in protein expression levels including decreases in EAAT2 expression by 29 % in the ipsilateral cortex 7 days post-injury (Goodrich et al. 2013). Additionally the use of antisense oligonucleotides to knockout expression of various subtypes of EAATs has indicated the transporters are crucial to maintaining concentrations below toxic levels. Knockout of either EAAT1 or EAAT2 in mice can produce excitotoxic levels of glutamate similar to that experienced following TBI and induce neuronal degeneration and loss of motor neuron viability (Rothstein et al. 1996). Similarly the introduction of knockdown antisense oligonucleotides for EAAT2 significantly increased hippocampal cell death compared to sense nucleotides and sham operated controls in a TBI model (Rao et al. 2001). Other studies have indicated comparable decreases in both subtypes of astrocytic glutamate transporters as well as their de novo expression in ramified microglia, possibly as a compensatory reaction to the increased extracellular glutamate levels and loss of transporter function (van Landeghem et al. 2001). Collectively, these studies demonstrate the crucial role of astrocytic EAATs, especially EAAT2, in the maintenance of extracellular glutamate within physiological concentrations and illustrate how the pathology of CNS injury can be exacerbated when function or expression of these transporters is compromised.

Although astrocytic EAATs are responsible for the majority of the removal of glutamate from the extracellular space, the neuronal EAATs are also affected by CNS injury. Increases in expression of EAAT4 have been reported in hippocampal astrocytes 3 to 7 days following TBI which may also be compensatory (Yi et al. 2007). This result is novel in that EAAT4 is typically expressed in neurons; particularly Purkinje cells, has a much higher affinity for glutamate, and has a unique chloride conductance that is not coupled to its glutamate transport function. The chloride ion conductance is believed to play a role in decreasing cellular excitability via influx of the ion resulting and cell hyperpolarization (Fairman et al. 1995). Thus, like the de novo expression of astrocytic glutamate transporters, the increased appearance of the typically neuronal EAAT4 in hippocampal astrocytes may represent an endogenous neuroprotective attempt to mitigate high extracellular glutamate levels following the loss of the primary removal mechanism (Yi et al. 2007). Other attempts to offset the loss of EAAT function following TBI have involved the use of therapeutic drugs that upregulate expression of the transporters. Goodrich and colleagues have indicated that intraperitoneal injections of the β-lactam antibiotic ceftriaxone reversed the loss of EAAT2 expression in the ipsilateral cortex of fluid percussion injured mice by 7 days post-injury and furthermore decreased astrocytic degeneration (as measured by GFAP expression) by 43 % when compared to ipsilateral cortex of non-treatment control mice (Goodrich et al. 2013).

Importantly in the acute response to CNS injury, the expression of EAATs appears to be modified on almost every level possible, from DNA transcriptional regulation, to mRNA splicing, protein synthesis, and post-translational modification

(Danbolt 2001). A primary means of regulating astrocytic expression of EAAT2 involves molecules secreted by neurons. As evidence of this concept, astrocytes cultured in the absence of neurons preferentially express EAAT1 with very little expression of EAAT2, while neuronal co-culturing with astrocytes increases expression of EAAT1 and induces the expression of EAAT2 (Danbolt 2001). This induction of EAAT2 in the presence of neurons appears to be the result of soluble factors given off by the neurons and not the result of direct cell-to-cell adhesion between astrocytes and neurons. Evidence supporting the idea that soluble factors represent a necessary and sufficient mediator of EAAT2 expression includes data demonstrating that the introduction of neuron-conditioned media to pure astrocyte cultures can induce EAAT2 expression (Gegelashvili et al. 1997). Additionally immunofluorescence staining of embryonic mouse cortical neurons demonstrates that the majority of EAAT2 containing glial cells are not in direct contact with neurons (Gegelashvili et al. 2000). The neuronal conditioned media induced-increases in EAAT2 expression can be mimicked by the addition of dibutyryl-cyclic AMP, epidermal growth factor (EGF), or pituitary adenylate cyclase-activating polypeptide (PACAP) (Swanson et al. 1997).These effects seem to be dependent on the activation of phosphatidylinositol 3-kinase (PI-3K) and nuclear transcription factor-κB (NF-κB), the latter of which has been demonstrated to directly regulate the glutamate transporter gene (Sitcheran et al. 2005). A key mediator between these two signaling molecules is the protein kinase Akt. Akt is activated by a number of growth factors and frequently functions downstream of PI-3K where it has the ability to increase NF-κB activity by decreasing the activity of the protein responsible for NF-κB's sequestration in the cytoplasm (Kane et al. 1999). Li and colleagues have demonstrated the importance of the Akt pathway in EAAT2 induction by using lentiviral vectors to create astrocytic cultures that express dominant-negative or constitutively active variants of Akt. The dominant-negative strain decreased the effects of EGF on EAAT2 expression while the constitutively active stain demonstrated a dose and time dependent increase in EAAT2 protein expression, mRNA levels, and transport activity thereby demonstrating that Akt can regulate the expression of EAAT2 by increasing its rate of transcription (Li et al. 2006).

Another method for EAAT induction by growth/neurotrophic factors involves preferentially targeting the receptor tyrosine kinase (RTK) pathway. Gegelashivili and colleagues have shown that inhibition of RTK by the cell permeable Typhostin A23 blocks the induction of EAAT2 in the presence of neuron-conditioned medium (NCM) (Gegelashvili et al. 2000). The RTK pathway appears to represent a primary starting point for a second messenger cascade, which converges on the mitogen-activated protein kinases (MAPK) p42 and p44, and ultimately results in increased expression of EAAT2. Binding of RTK by growth factors results in recruitment of the GTP-binding protein Ras and the stimulation of MAPK kinase kinases like Raf, which activates MAPK kinases MEK1 and 2, that in turn activate p42/p44 MAPK (Abe and Saito 2001). A crucial prerequisite of EAAT2 induction involves this phosphorylation of p42/p44 MAPKs. The double phosphorylation of p42/p44 MAPKs at threonine-202 and tyrosine-204 has been shown to correlate with expression of EAAT2 in the presence of NCM (Gegelashvili et al. 2000).

Phosphorylated p42/44 MAPKs can translocate to the nucleus and regulate gene transcription through activation of transcription factors like cyclic AMP (cAMP) responsive element binding protein (CREB) (Abe and Saito 2001). Growth factors can bypass the RTK-p42/44 MAPK pathway and still influence the expression of EAAT2 by directly activating transcription factors such as cAMP responsive element modulator, CREB, and activating transcription factor 1, although the induction of EAAT2 via these pathways is weaker than through the RTK controlled pathways. Additionally, the growth factor activation of PI-3K previously mentioned can lead to the activation not only of Akt, but also result in increased phosphorylation of p42/44 MAPKs demonstrating a convergence point in these two EAAT2 regulating pathways (Abe and Saito 2001). Further evidence supporting the importance of the p42/44 MAPK signaling pathway in the expression and regulation of EAAT2 comes from studies examining pure cultured cortical astrocytes exposed to varying concentrations of glutamate. Western blot analysis indicated that at increased concentrations of extracellular glutamate there were increased levels of phosphorylated p42/44 (Abe and Saito 2001). The changes in phosphorylated proteins occurred in a time and concentration dependent manner and did not induce changes in the total amount of cellular p42/44 (Abe and Saito 2001). Glutamate receptor agonists did not mimic the effects of glutamate-induced increases in phospho-p42/44, nor did glutamate receptor antagonists block them. However the effects of glutamate could be reproduced by molecules that could be transported into the cell by glutamate transporters; thus introducing aspartate or the transportable uptake inhibitors DL-*threo*-β-hyroxyaspartate (THA) and L-*trans*-pyrrolidine-2,4-dicarboxylate (PDC) into the extracellular environment lead to increases in phosphorylated p42/44 almost as strongly as glutamate itself. Together, these results indicate that EAATs are capable of modifying their own expression in response to extracellular glutamate independently of the activity of glutamate receptors. Thus, the reuptake transporters are able to relay signals about the extracellular concentration of glutamate and activate second messenger systems leading to the recruitment of the p42/44 MAPK pathway and ultimately resulting in increases in the number of the transporters on the cell membrane (Abe and Saito 2001).

While RTK, Akt, and MAPK intracellular signaling molecules are predominantly involved in increases in EAAT expression and presence in the plasma membrane, glutamate transporters expression can also be regulated either through sequestration in intracellular storage sites or ubiquitin-mediated degradation. Of primary importance in selective EAAT downregulation is the activity of the signaling molecule protein kinase C (PKC). PKC has differential effects on the EAAT subtypes; in mixed neuronal and astrocyte cell cultures, the activation of PKC with phorbol ester caused a rapid (within minutes) decrease in cell-surface expression of EAAT2 (Kalandadze et al. 2002) and in increase surface expression in the neuronal glutamate transporter EAAT3 (Gonzalez and Robinson 2004). These differential effects are thought to represent a switching mechanism from astrocytic to neuronal glutamate uptake. However, as astrocyte transport represent the primary reuptake mechanism, the effect in a mixed cell culture would be overall reduced reuptake of glutamate and elevated extracellular levels of the neurotransmitter (Gonzalez and Robinson

2004). The deletion of amino acids 475–517 on EAAT2 abolishes the effects of phorbol ester-induced internalization, demonstrating a possible site of PKC phosphorylation on the transporter (Kalandadze et al. 2002). The decrease in cell-surface expression did not correspond with a reduction in total cellular levels of EAAT2, suggesting the immediate effect of PCK phosphorylation of EAAT2 involves internalization of the transporter to an intracellular sequestration site (Gonzalez and Robinson 2004). Internalization can be blocked in astrocyte cultures expressing a dominant-negative variant of clathrin (Susarla and Robinson 2008; Sheldon et al. 2008) or by inhibition of the ubiquitin enzyme E1 (Martinez-Villarreal et al. 2012), suggesting an ubiquitin-dependent, clathrin-mediated endocytic mechanism of sequestration. In contrast to short-term activation of PKC, long-term exposure to phorbol ester was accompanied by an overall decrease in total cellular EAAT2 expression (Susarla and Robinson 2008). This decrease was attenuated by lysosomal inhibitors, suggesting a cellular mechanism by which PKC can reduce EAAT2 levels under physiological or pathological conditions (Susarla and Robinson 2008). Indeed the PKC induced lysosomal degradation of EAAT2 could partially account for the rapid reduction in protein expression levels following TBI. Studies in astrocyte cultures have indicated that the half-life of EAAT2 is longer than 24 h (Zelenaia and Robinson 2000); therefore the observed decreases in EAAT2 expression in the hours immediately following TBI (Rao et al. 1998) cannot be accounted for solely by halting transcription of the transporter. Along with the aforementioned degradation of EAAT2 by caspase-3, the activity of PKC could possibly represent a mechanism by which the secondary injury phase of TBI induces dysfunction in glutamate reuptake transporters and exacerbate the deleterious effects of elevated extracellular glutamate.

## 6.4 Glutamate Receptors and Calcium Homeostasis

Under both physiological and pathophysiological conditions, glutamate acts on both ionotropic and metabotropic receptors. Glutamate binding to ionotropic receptors, particularly the N-methyl-D-aspartate (NMDA) receptor alters membrane permeability to a number of ions including sodium ($Na^+$), potassium ($K^+$) and calcium ($Ca^{2+}$) (Arundine and Tymianski 2003). The neurotoxic effects of TBI appears to be largely the result of NMDA receptor activation as blockage of the NMDA receptor attenuates some of the neurotoxic effects of TBI, especially by influencing calcium homeostasis (Demediuk et al. 1989; Faden et al. 1989b). In a seminal set of experiments, Choi and colleagues discriminated the neurotoxic effects of the calcium and sodium ions on neuronal survival by examining cell cultures exposed to toxic levels of glutamate in isolated extracellular environments (Choi 1987). In an extracellular environment that mimicked baseline physiological concentrations of both sodium and calcium, cells exhibited immediate morphological changes followed later by significant cell degradation and death. Removal of both ions from the extracellular environment showed large protective effects on the cells, even at prolonged

glutamate exposure. However, separation of the two ions indicated their unique roles in the excitotoxic cascade. Their results indicate that the acute event precipitated by sodium appears to be transient and largely non-toxic to cells. Cultures in a calcium free extracellular environment exhibited morphological changes including swelling and granulation to a greater degree than those in the baseline experiment, but were largely spared later cell death and returned to previous size within an hour (Choi 1987). Cultures in an environment where choline was substituted for sodium and calcium ions remained at physiological concentrations showed markedly less swelling and other morphological changes than those at baseline levels, but exhibited roughly the same amount of cell death as cultures with both ions at physiological levels (Choi 1987).

Metabotropic glutamate receptors (mGluRs) can also mediate changes in intracellular ion concentrations. These receptors are G- protein-couple receptors that signal via second messenger system including phosphoinositide-dependent processes, cyclic AMP, or PKC. Three groups of mGluRs have been characterized with group I mGluRs (mGluR1 and mGluR5) being positively coupled to phospholipase C (PLC) to induce phosphoinosidide hydrolysis and mobilization of intracellular calcium. Groups II and III are negatively coupled to adenylyl cyclase. Thus the group I mGluRs are thought to have the most direct role in modulating intracellular calcium after injury (Lea and Faden 2001). With regard increases in astrocytic intracellular calcium after injury, in vitro mechanical strain injury was shown to increase inositol trisphosphate ($IP_3$) up to 10-fold over uninjured controls acutely post injury. Importantly, pharmacological inhibition of PLC was shown to inhibit injury-induced increases in IP3 and the resultant increase in intracellular calcium (Floyd et al. 2001). It was subsequently shown that pharmacological inhibition of the group I mGluRs inhibited injury-induced increases in PLC and increases in astrocytic intracellular calcium (Floyd et al. 2004). Similarly, administration of a group I mGluR antagonist was shown to confer cellular protection and reduction in injury-induced learning and memory deficits in a rat model of TBI (Gong et al. 1995). This finding is mirrored in spinal cord injury as antagonism of the group I mGluRs was shown to increase tissue sparing and improve locomotor scores (Mills et al. 2002).

Regardless of the glutamate receptor type that induces pathological elevations in intracellular calcium, there are a number of immediate consequences of excessive influx of calcium into the intracellular space. Calcium entry to the cell directly activates a family of nonlysosomal, cysteine proteases, calpains, which bind calcium and begin to enzymatically cleave the αII-spectrin protein, a key component of the cytoskeletal architecture resulting in cellular degeneration (Saatman et al. 2010). Prolonged calpain levels have been indicated in necrotic degeneration associated with CNS injury (Wang 2000). Calpains are one of the earliest mediators of cellular death in the injury pathology with elevated levels of the proteins demonstrated as early as 15 min after injury, indicating that calpains may underpin a an early response to injury (Zhao et al. 1998). Other proteolytic enzymes activated by TBI include the caspases, a family of 14 cysteine proteases discovered to be related to gene products required for cellular apoptosis in the nematode (Thornberry 1997). Caspases function as either initiators, which respond to apoptotic cellular signaling, or effectors, which

directly participate in cellular degradation largely through the cleavage of multiple proteins responsible for proper cellular functioning (Thornberry 1997). Caspase levels increase following TBI while caspase inhibitors have been demonstrated to reduce apoptosis and neurological deficits following TBI (Raghupathi 2004). Additional reports have indicated that a specific effector caspase, caspase-3, is responsible for degradation of the principle glutamate transporter, EAAT2, by cleavage near the C-terminal domain at aspartate-505 resulting in significant loss of function (Boston-Howes et al. 2006). This loss of transporter function can further exacerbate the excitotoxic effects of elevated glutamate levels in the extracellular space and serve to prolong and exacerbate glutamate-mediated pathology.

While activation of calpains and caspases represent immediate reactions to apoptotic and necrotic signaling, delayed responses to the CNS injury include changes in genes involved in cell death and survival, including the *blc-2* gene family which codes for both pro-apoptotic proteins Bax and Bad and the pro-survival protein Blc-2 (Strauss et al. 2004). Following injury, increased mRNA levels of the anti-apoptotic gene *blc-2* are present in surviving neurons as early as 6 h after injury (Clark et al. 1997), while transgenic mice that overexpress the *blc-2* gene exhibit less neuronal degeneration after the injury (Raghupathi et al. 1998). Additionally in regions exhibiting increased loss of neurons, mRNA levels of *blc-2* were markedly decreased, while those of the pro-apoptotic *bax* gene were significantly increased (Strauss et al. 2004), demonstrating the role of the *blc-2* family and the ratio of the proteins that they code for are key in determining the fate of neurons following injury. Part of the determination of this ratio involves post-translational modification of several downstream proteins by Akt to promote cell survival (Zhang et al. 2006). Akt has the ability to phosphorylate Bad at serine-136 both in vitro and in vivo, inhibiting the ability to participate in the apoptotic pathway (Datta et al. 1997). Akt is itself regulated by phosphorylation at serine-473 and threonine-308. Decreased levels of phospho-Akt have been shown 1 h after TBI in the injured cortex, correlating with an increase in cell death, however in surviving neurons, phosphorylation of Akt was rapidly accelerated after 4 h post injury to encourage cell survival (Noshita et al. 2002). Phospho-Akt has a number of downstream effectors, including forkhead family transcription factors, andCREB, activation of these and other proteins by Akt shifts the balance of pro- and anti-apoptotic enzymes in favor of cell survival (Zhang et al. 2006).

Pro-apoptotic signaling pathways are also activated in response to the myriad of toxic stimuli activated during the secondary injury phase of CNS injury. One signaling molecule appearing to have primary importance for apoptosis in neuronal tissues is the c-Jun N-terminal Kinase (JNK). JNKs are phosphorylated and activated in response to DNA damage, axonal injury, and the formation of reactive oxygen species, all of which are present in the secondary injury phase. JNKs appear to represent a convergence point for a number of signaling molecules including MAPKs and mixed-lineage kinases (Xu et al. 2001). Additionally the activation of Akt appears to inhibit the activity of JNK, reducing its apoptotic influence while inhibition of JNK using geldanamycin reduces its activity and provides neuroprotection both

in vitro and in vivo (Kovesdi et al. 2007). Another pro-apoptotic protein, p53 also appears to increase its activity following DNA damage in TBI models. Elevated levels of p53 mRNA and associated protein levels have been reported in experimental models of TBI (Napieralski et al. 1999). These studies demonstrate that the secondary injury phase induces important changes in signaling molecules responsible for cell death and survival. As these changes in molecular signaling take place over an extended period following the injury, they represent potent targets for potential therapeutic intervention.

## 6.5 Role of Astrocytic Sodium and Potassium Regulation in Pathology

Clearly, intracellular calcium is a key component in the glutamate pathology in the astrocyte after CNs injury. However, other work has established that in the astrocyte, $Na^+$ and $K^+$ are also key modulators of glutamate-induced damage after injury. For example, using an in vitro model of strain-induced cellular injury, Floyd and colleagues (Floyd et al. 2005) demonstrated that either injury or exogenous glutamate application induced elevations of astrocytic intracellular $Na^+$ that were depended on injury severity and that these increases in intracellular $Na^+$ were significantly reduced, but not completely eliminated, by inhibition of glutamate uptake. Moreover, pharmacological blockade of the reversed mode of the sodium-calcium exchanger reduced intracellular calcium concentrations and astrocyte death after injury (Floyd et al. 2005). Similarly, in an in vivo model of TBI, pharmacological inhibition of the sodium-calcium exchanger or the type 1 sodium-proton antiporter were found to reduce astrocyte and neuronal death acutely after injury (Zhao et al. 2008). Taken together, these data indicate that astrocytic increases in intracellular sodium, particularly those associated with glutamate uptake, also contribute to injury-induced pathology.

Additionally, a growing body of evidence suggests that alteration in $K^+$ influx in astrocytes is critical to CNS injury pathology. It is well established that the highly negative resting membrane potential and high $K^+$permeability of astrocytes is maintained by the inwardly rectifying $K^+$ channel Kir4.1. Moreover, Kir4.1 is the channel that supports potassium spatial buffering and homeostasis, both vital astrocyte functions that maintain normal neuronal firing (Olsen et al. 2006, 2007. Kir4.1 also affects astrocyte clearance of glutamate released by neurons. Actually, nearly all glutamate uptake is achieved by sodium-dependent glutamate transporters on astrocytes that are electrogenic and more effective at negative membrane potentials. Thus, alteration in Kir4.1 signaling could have large implications for astrocyte-neuronal interactions in the normal or injured spinal cord (Olsen et al. 2010). Additional clues to the role of Kir4.1 in pathology come from the relationship between channel function and cell proliferation/differentiation. Previous work has shown that dividing and immature cells have a relatively positive resting membrane potential

that becomes more negative as cells mature and terminally differentiate and this transition is associated with the increased expression of Kir4.1 channels (Olsen et al. 2006, 2007). Several lines of evidence substantiate this relationship between Kir4.1 expression and cell division. For example, blockade of the Kir4.1 channel delays differentiation and exit from the cell cycle (MacFarlane and Sontheimer 2000), and glia in Kir4.1 knock-out mice display depolarized resting membrane potentials and immature morphologies (Djukic et al. 2007; Neusch et al. 2001). Also, malignant human glioma cells maintain a relatively depolarized membrane potential and have mislocalized/non-functional Kir4.1 channels, but transfection with functional Kir4.1 channels hyperpolarizes the cell membrane and arrests cell division (Olsen et al. 2006, 2007). With regard to injury, *in vitro* injury to spinal cord astrocytes decreased Kir4.1 activity and induced proliferation (MacFarlane and Sontheimer 1997). TBI was shown to induce abnormal $K^+$ accumulation that was similar to that achieved by blockade of Kir4.1 channels (D'Ambrosio et al. 2002). Similarly, it was shown in an SCI model that Kir4.1 expression and function was reduced by nearly 80 % after injury and that this down-regulation extended to spinal segments several millimeters from the lesion epicenter. Moreover, the loss of Kir4.1 was accompanied by functional loss of astrocytic glutamate transport (Olsen et al. 2010).

## 6.6 Summary

Traumatic brain and spinal cord injury are life-changing events that exact an enormous financial and societal toll. Currently, there are no widely accepted and clinically-approved treatments to reduce damage after traumatic CNS injury, making the development of novel treatments an important health care priority. Astrocytes play a critical role in the pathophysiology of traumatic CNS injury and therefore also represent potential therapeutic targets. Key components in the astrocytic response include astrocytic glutamate transporters, glutamate receptors, and modulators of calcium, sodium, and potassium homeostasis in the astrocyte. Indeed, a combination approach that targets several astrocytic mechanisms may also provide great therapeutic potential.

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