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**Excitotoxicity and Na⁺-dependent Glutamate Transport in Spinal Cord
White Matter Injury**

By

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**This thesis is submitted as a partial fulfillment of the Ph.D. program
in Cellular and Molecular Medicine Graduate Program**

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Contributions of each co-author in the publication

All the surgical preparations, electrophysiology of compound action potentials, immunohistochemistry, laser confocal imaging and image processing were performed by myself. Glutamate fluorescence measurements and quantitative data analysis were performed by Dr. Peter Stys. Patch clamp recording in cultured neurons in *Chapter 4* was conducted by Geoff Mealing and Dr. Paul Morley.

ABSTRACT

Spinal cord injury (SCI) is a devastating condition, with much of the clinical disability resulting from disruption of ascending and descending white matter tracts. Recent reports suggest that a component of axonal dysfunction during SCI involves glutamate-mediated white matter damage, but the cellular targets of excitotoxicity and the precise mechanisms of glutamate release from non-synaptic white matter are not understood. In the present study, using combined techniques including electrophysiology, pharmacology, immunohistochemistry and confocal microscopy, we demonstrate that myelinated axons in isolated dorsal columns are vulnerable to irreversible excitotoxic injury, which is primarily dependent on Ca^{2+} -permeable AMPA receptors. The cellular components susceptible to glutamate include oligodendrocytes, astrocytes and the myelin sheath, consistent with the distribution of GluR3 and GluR4 in these cell types *in situ*, but not GluR2. We also demonstrate that reduced transmembrane Na^+ and K^+ gradients induced by inhibiting Na^+ - K^+ -ATPase with ouabain plus high K^+ could drive Na^+ -dependent glutamate transporters to operate in a reverse mode, resulting in glutamate release from intracellular compartments and functional failure in white matter tracts by activation of AMPA receptors. Using injury models of anoxia or trauma, we further show that the ionic and membrane potential perturbations induced during *in vitro* anoxia or trauma are sufficient to cause toxic glutamate efflux via reverse Na^+ -dependent glutamate transport, resulting in damage to the myelin sheath and possibly other structures by activation of AMPA receptors. Semiquantitative measurement of intracellular glutamate indicates that axon cylinders, and to a lesser extent oligodendrocytes, are the major

cellular sources of endogenous glutamate release. Our findings are consistent with the immunolocalization of Na⁺-dependent transporters (GLT1, EAAC1 and GLAST) in dorsal columns. We conclude that white matter, especially glial elements including myelin, is vulnerable to excitotoxins acting via AMPA receptors; release of glutamate by reversal of Na⁺-dependent glutamate transport with subsequent activation of these receptors is an important mechanism in anoxic and traumatic injury of spinal cord white matter.

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List of Abbreviations

ACSF	artificial cerebrospinal fluid
AMPA	α -amino-3-hydroxy-5-methylisoxazolepropionic acid
$[\text{Ca}^{2+}]_i$	intracellular Ca^{2+}
CAP	compound action potential
CNPase	2',3'-cyclic-nucleotide 3'-phosphodiesterase
CNQX	6-cyano-7-nitroquinoxaline
CNS	central nervous system
CTZ	cyclothiazide
EAAC1	excitatory amino acid carrier 1
EAAT	excitatory amino acid transporter
EGTA	ethylene glycol-bis(b-aminoethyl ether)-N,N,N',N'-tetraacetic acid
EPSP	excitatory postsynaptic potential
ER	endoplasmic reticulum
GFAP	glial fibrillary acidic protein
GLAST	glutamate aspartate transporter

GLT1	glutamate transporter 1
GluR	glutamate receptor
Glut	glutamate
G-protein	GTP-binding protein
GYKI52466	1-(4-aminophenyl)-4-methyl-7, 8-methylenedioxy-5H-2, 3-benzodiazepine
GYKI53655	1-(4-aminophenyl)-4-methyl-7, 8-methylenedioxy-5H-2, (3N methylcarbamate)-2, 3-benzodiazepine
H ₂ O ₂	hydrogen peroxide
HEK 293	human embryonic kidney
IP ₃	inositol 1,4,5-trisphosphate
JSTX	Joro spider toxin
KA	kainate
KB-R7943	2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl] isothiourea methanesulfonate
KYN	kynurenic acid
LAOBP	lysine-arginine-ornithine-binding protein

LTP	long-term potentiation
mGluR	metabotropic receptor
MK-801	methyl-10, 11-dihydro-5-H-dibenzocyclohepten-5,10-imine
MPT	mitochondrial permeability transition
NAD	nicotinamide adenine dinucleotide
NBQX	1,2,3,4-tetrahydro-6-nitro-2,3-dibenzo[f]quinoxaline-7-sulfonamide
NGS	normal goat serum
NMDA	N-methyl-D-aspartate
NO	nitric oxide
$O_2^{\bullet -}$	superoxide anion radical
$OH^{\bullet}$	hydroxyl radical
$ONOO^-$	peroxynitrite anion
PARP	poly (ADP-ribose) polymerase
PBS	phosphate-buffered saline
Pf	fractional Ca^{2+} current

SBP	spectrin breakdown product
SCI	spinal cord injury
SOD	superoxide dismutase
Trans-PDC	L-trans-pyrrolidine-2,4-dicarboxylic acid
TTX	tetrodotoxin
TUNEL	terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling
VGCC	voltage-gated Ca^{2+} channel

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Chapter 1

General Introduction

In the central nervous system (CNS), axons are critically important for transmitting information between neurons with high fidelity and reliability. Disruption of the axonal tracts, a common feature of many CNS disorders, such as ischemia, trauma and multiple sclerosis, results in significant morbidity and mortality. Spinal cord injury (SCI), an unexpected catastrophic event, is one of the most serious of such disorders principally due to the dysfunction of white matter tracts rather than gray matter regions (Blight and Decrescito, 1986; Fehlings and Tator, 1995; Noble and Wrathall, 1989). The consequences of SCI may produce clinical disability, which usually persists for the life of the patient. This devastating insult mainly affects young adults during the prime productive years of their lives. Thus, SCI may cause a huge personal and socioeconomic burden on society because of the high costs of hospitalization plus the subsequent expensive supportive care. Though some prevention programs have been initiated, there is no evidence that the incidence of this disease is declining (Wright et al., 1995). Therefore, development of effective treatments for SCI is of importance, and would produce great benefits, if successful.

Traumatic SCI is a particularly striking example of severe disability as a result of permanent dysfunction of central axons. Following SCI, the neurological disability of the patient is not significantly determined by the local gray matter changes, but rather by the

interruption of long white matter pathways. A relatively small volume of white matter tract damage can lead to devastating clinical consequences, and the neurological state of paraplegic or quadriplegic patients is largely determined by the residual number of white matter fibers following traumatic injury. However, clinically, it is unusual for the spinal cord to be completely transected during trauma caused by motor vehicle, sporting or industrial accidents, the most common causes of SCI. Actually, a large proportion of patients have anatomical continuity of the spinal cord at the level of lesions, indicating relative sparing of some axons even in severe SCI (Bunge et al., 1993; Bunge et al., 1997; Kakulas et al., 1998). Therefore, it becomes a key issue to properly protect the function of anatomically connected axons following SCI, which are usually subjected to a prolonged impairment by a variety of secondary injury mechanisms following the primary insult (Li and Tator, 1998; Tator, 1991; Tator and Koyanagi, 1997).

So far, a large number of pharmacological neuroprotective agents have been examined targeting the residual, but functionally disrupted tissues after SCI (Taoka and Okajima, 1998). Unfortunately, only very few of them, such as methylprednisolone and GM1 ganglioside, have clinically been shown to provide very limited protection against delayed tissue impairment after acute SCI (Bracken et al., 1990; Bracken et al., 1997; Geisler et al., 1991; Young, 1996; Young et al., 1994). The lack of effective treatment for SCI is principally attributed to the limited understanding of cellular and subcellular injury mechanisms of central myelinated axons. Therefore, from a therapeutic perspective, it is of great importance to study the precise mechanisms of how the traumatic or anoxic/ischemic damage is initiated and exacerbated during axonal injury.

1.1. Cellular and molecular injury mechanisms of CNS myelinated axons

Much work has been done on the pathophysiology of gray matter traumatic and anoxic/ischemic injury, particularly the latter, involving various mechanisms including cellular Ca^{2+} overload occurring largely through glutamate-mediated receptors and possibly voltage-gated Ca^{2+} channels, cell swelling as a result of excessive Na^+ and Cl^- influx, free radical production, and delayed apoptotic cell death (Lee et al., 1999; Lipton, 1999). In contrast, much less is known about the fundamental mechanisms of anoxic/ischemic and traumatic injury to CNS myelinated axons, though white matter has been shown to be very vulnerable to these kinds of injury (Follis et al., 1993; Pantoni et al., 1996). The structural and functional similarities between gray and white matter, such as ion channels, transporters, enzymes, Ca^{2+} dynamics and mitochondrial physiology, indicate that both tissues may share at least some common mechanisms. For instance, following Ca^{2+} entry into cells, the downstream damage mechanisms in the two types of tissue largely overlap, such as excess activation of Ca^{2+} -dependent biochemical pathways, mitochondrial injury and apoptotic cell death. However, the unique architecture of white matter (see below) portends the specialized pathophysiological features distinct from those of gray matter. A number of studies support that glutamate-mediated ion channels and voltage-gated Ca^{2+} channels (VGCC) significantly contribute to gray matter pathophysiology by allowing excessive Na^+ and Ca^{2+} influx, whereas the major ion flux pathways of Na^+ and Ca^{2+} in myelinated axons are significantly different (for review see Stys, 1998). Therefore, it is necessary to study the fundamental cellular injury

mechanisms of white matter tissue, a thorough understanding of which is essential for rationally designing efficient neuroprotectants aimed at injured axons.

Myelinated axons possess a unique structure including the highly specialized axonal ion channels and myelin sheath (Salzer, 1997; Waxman and Ritchie, 1993). The ion channels and transporters, which produce the required ionic currents for the generation and propagation of action potentials, are highly segregated on the axon membrane. Nodes of Ranvier display a high density of voltage-gated Na^+ channels at about $1000\text{-}2000/\mu\text{m}^2$ (Waxman, 1995). K^+ channels, present primarily on the internodal axolemma, contribute to the maintenance of hyperpolarized resting membrane potentials (Bostock and Grafe, 1985; Chiu and Ritchie, 1982). $\text{Na}^+\text{-K}^+\text{-ATPase}$, the major active ion transporter for keeping adequate transmembrane Na^+ and K^+ gradients, plays a key role in maintaining physiological functions of myelinated axons. Taken together, the integrative role of these structures makes it possible to conduct action potentials in a very efficient manner. The axonal ion channels conduct the ionic currents efficiently, while myelin provides electrical insulation to avoid trans-axolemmal current leaks, allowing rapid and reliable conduction along the myelinated axons. Moreover, maintaining intact function of myelinated axons is highly dependent on a stable and intimate relationship with surrounding glial cells, such as the cell bodies and processes of astrocytes, oligodendrocytes, since these cells contribute to the integrative functions of myelinated axons by providing metabolic support and are involved in the formation of the insulating sheath. Thus, disruption of any of these structures mentioned above may result in partial

or complete failure of action potential conduction, which occurs frequently in the setting of traumatic and ischemic injury of white matter tracts.

Following trauma, such as acute SCI, two temporally distinct injury mechanisms have been implicated: the primary mechanical injury, and a secondary injury due to several additional damaging processes initiated by the primary event (Collins, 1983; Sandler and Tator, 1976). The pathophysiological processes involving the secondary injury cascades include vascular, electrolyte and biochemical changes, cell edema and loss of energy metabolism (Li and Tator, 1998; Tator and Koyanagi, 1997), which are quite similar to those occurring during anoxic/ischemic injury. Indeed, a series of studies has recently elucidated the relevance of many basic mechanisms of white matter injury resulting from trauma and anoxia/ischemia at the cellular level. The cellular damage caused by either anoxic/ischemic or mechanical injury to CNS white matter is heavily dependent on abnormal Na^+ influx through voltage-gated Na^+ channels, intracellular Ca^{2+} overload and glutamate excitotoxicity (Agrawal and Fehlings, 1997; Fern et al., 1995; Garthwaite et al., 1999; Imaizumi et al., 1997; Imaizumi et al., 1999; Lopachin, 1999; Stys, 1998; Stys et al., 1992a; Wrathall et al., 1997), indicating the possibility of applying the concepts from one area to try to understand injury mechanisms to the other. Whether due to anoxia or trauma, the common injury mechanisms of myelinated axons mainly include the excessive accumulation of Ca^{2+} ions in intracellular compartments, excessive formation of free radicals, damage to mitochondria, delayed apoptosis, which are briefly reviewed in this section.

1.1.1 Ca^{2+} overload

Ca^{2+} is an essential messenger in a variety of cellular processes. The major requirement for the function of Ca^{2+} signaling is to maintain large concentration gradients of 4-5 orders of magnitude across the plasma membrane. Under physiological conditions, the levels of intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) are tightly regulated by several mechanisms consisting of entry into or extrusion from the cytoplasm, and uptake into or release from internal stores (Fig.1). Most neural cells exhibit a variety of voltage-gated Ca^{2+} channels (Barres et al., 1988; Sontheimer et al., 1996), and Ca^{2+} influx via these channels significantly increases $[\text{Ca}^{2+}]_i$ in these cells (Steinhauser and Gallo, 1996; Verkhratsky and Kettenmann, 1996). A number of ligand-gated ionotropic receptors, such as ionotropic glutamate receptors expressed in neuronal and glial cells, are also responsible for Ca^{2+} influx. The Na^+ - Ca^{2+} exchanger and ATP-dependent Ca^{2+} pump, present on the membrane of neuronal somata, axon cylinders and glia, play a key role in extruding Ca^{2+} across the plasmalemma (Finkbeiner, 1993; Goldman et al., 1994; Kirischuk et al., 1997). Meanwhile, internal Ca^{2+} stores also significantly contribute to maintaining Ca^{2+} homeostasis within cells through release from or uptake into Ca^{2+} stores. The major mechanisms for Ca^{2+} release from internal stores, particularly endoplasmic reticulum (ER), involve activation of inositol 1,4,5-triphosphate (IP_3)-gated Ca^{2+} channels (IP_3 receptors) present in most neural cells (Khodakhah and Ogden, 1993; Yamamoto-Hino et al., 1995). IP_3 is produced by activation of phospholipase C coupled via G proteins to plasmalemmal metabotropic receptors. Another type of Ca^{2+} -gated channel (ryanodine receptor), sensitive to the classical modulators ryanodine and caffeine, may also significantly contribute to Ca^{2+} release from internal stores (Furuichi et al., 1994;

Meissner, 1994)]. In addition, mitochondria, another capacious Ca^{2+} storage site, significantly contribute to maintaining cellular Ca^{2+} homeostasis by uptake from or release into the cytosol (Simpson and Russell, 1998). Mitochondria, especially those located close to plasma membrane or ER, where high Ca^{2+} microdomains occur, are capable of taking up substantial amounts of Ca^{2+} via the Ca^{2+} uniporter when the overall $[\text{Ca}^{2+}]_i$ increases. They can also contribute to cytosolic Ca^{2+} by releasing this ion via Na^+ - Ca^{2+} exchange, and possibly the permeability transition pore (Simpson and Russell, 1998). Taken together, by modulating membrane Ca^{2+} pathways and internal Ca^{2+} stores, neural cells are able to sense and react to various neurotransmitters, evoking $[\text{Ca}^{2+}]_i$ changes, the essential messengers for maintaining physiological functions of these cells.

Under a variety of pathological conditions, the changes of $[\text{Ca}^{2+}]_i$ are generally more pronounced and sustained, and the excessive elevation of $[\text{Ca}^{2+}]_i$ can be very harmful to the cells, even causing cell death. In the CNS, Ca^{2+} -induced cytotoxicity has been shown to play a major role in several neuropathological conditions such as stroke, trauma and chronic neurodegenerative diseases, including damage to white matter tissues. Following acute axonal injury, abnormal fluxes of monovalent ions occur, and are responsible for the acute early failure of axonal excitability. But loss of electrical excitability as a result of collapse of Na^+ and K^+ gradients appears insufficiently to induce irreversible injury of cells. A 10-15 min anoxia to rat optic nerve, the CNS myelinated axonal tract, completely abolishes excitability and causes marked loss of axoplasmic K^+ , but function of this white matter tract recovers completely following reoxygenation (LoPachin and Stys, 1995; Stys, 1998). However, a number of studies elucidate that cellular Ca^{2+} overload is a key

and fundamental step in a series of events leading to injury under a variety of conditions including anoxia/ischemia or trauma (Kristian and Siesjo, 1998; Stys, 1998; Tymianski and Tator, 1996), and thus, the excessive Ca^{2+} accumulation is thought to be the “final common pathway” of necrotic cell death (Nicotera et al., 1992; Siesjö, 1986; Stys, 1998). During a 60 min anoxic period, the degree of optic nerve injury has been shown to be proportional to the Ca^{2+} in bath and to the time during which the anoxic tissue is maintained in high Ca^{2+} . The injury could be completely prevented by removing Ca^{2+} from the perfusate (Stys et al., 1990). Ultrastructural studies indicate that removal of Ca^{2+} largely protects the structural integrity of axonal cytoskeleton, except mitochondrial swelling (Waxman et al., 1993). Measuring axoplasmic Ca^{2+} of optic nerve further supports a substantial and continuous influx of Ca^{2+} during white matter anoxia (LoPachin and Stys, 1995). In the isolated dorsal column, several studies confirm the important role of extracellular Ca^{2+} in anoxic and traumatic injury of the white matter tract (Agrawal and Fehlings, 1998; Imaizumi et al., 1997; Imaizumi et al., 1999). Therefore, excess Ca^{2+} accumulation into axoplasm, largely from influx across plasma membrane, is a pivotal event in induction of anoxic and traumatic injury of CNS white matter.

Generally, Ca^{2+} may enter a cell across its plasma membrane through several routes: 1) VGCCs, 2) ligand-gated ion channels, 3) ion-coupled transporters such as reverse Na^+ - Ca^{2+} exchanger or 4) leakage through the channels mainly permeable to other ions or non-specific leakage through cell membrane (Stys, 1998; Verkhratsky et al., 1998). In the neuronal soma and dendrites, excessive Ca^{2+} entry via N-methyl-D-aspartate (NMDA)

and α -amino-3-hydroxy-5-methylisoxazolepropionic acid (AMPA) receptors has been shown to be an important route in neuronal injury (Choi, 1992a; Choi, 1992b). In contrast, Ca^{2+} influx through reversed Na^+ - Ca^{2+} exchange or voltage-gated Ca^{2+} channels appears to play a central role in Ca^{2+} accumulation of white matter. Experiments on optic nerve as well as dorsal column indicate that influx of extracellular Na^+ is important for anoxic and traumatic injury since replacing Na^+ with choline, Li^+ , or N-methyl-D-glucamine significantly improves the recovery of conduction after 60 min anoxic or compressive axonal injury (Agrawal and Fehlings, 1996; Imaizumi et al., 1997; Stys et al., 1992a). Together with the dependence of the injury on external Ca^{2+} , these findings indicate that Na^+ and Ca^{2+} movement may be linked, probably through Na^+ - Ca^{2+} exchange. Indeed, experiments showed that Na^+ -depletion largely prevents axoplasmic Ca^{2+} accumulation in anoxic axons (Lehning et al., 1996), and electrophysiological studies using inhibitors of Na^+ - Ca^{2+} exchange (Imaizumi et al., 1997; Li et al., 2000; Stys et al., 1992b) provide evidence for this antiporter as the major mechanism of Ca^{2+} overload in anoxic and traumatic white matter injury. These findings are congruent with localization of this ion transporter in central myelinated axonal tissues of optic nerve and spinal cord (Steffensen et al., 1997). In addition to the role of Na^+ - Ca^{2+} exchange, VGCCs, probably N- and L-type Ca^{2+} channels, have electrophysiologically been shown to contribute to permeating significant amounts of Ca^{2+} during anoxia of CNS myelinated axons (Agrawal and Fehlings, 1998; Fern et al., 1995; Imaizumi et al., 1999), though these results contrast to those of ion measurement experiments (Stys and LoPachin, 1998).

Taken together, the impairment of energy metabolism during anoxia/ischemia or trauma (Liaw et al., 1985; Vink et al., 1988) due to deprivation of oxygen and/or glucose results in the drop of ATP levels, inducing the failure of ATP-dependent pumps including axolemmal $\text{Na}^+\text{-K}^+\text{-ATPase}$ and endoplasmic reticulum $\text{Ca}^{2+}\text{-ATPase}$. The failure of these pumps may cause accumulation of Na^+ into and loss of K^+ from the intracellular space. The elevation of internal Na^+ mediated by TTX-sensitive channels, particularly the noninactivating subtype (Stys et al., 1993), together with the depolarization due to K^+ loss, stimulates reverse operation of $\text{Na}^+\text{-Ca}^{2+}$ exchanger and elicits deleterious amounts of Ca^{2+} accumulation in the cells. It is notable that intracellular Ca^{2+} stores may also be a potential source of $[\text{Ca}^{2+}]_i$ elevations in white matter injury (Ren and Stys, 1999), but more investigations are required to confirm this. The overload of intracellular Ca^{2+} may activate various Ca^{2+} -dependent enzymes including proteases, phospholipases, endonucleases and protein kinase C, generate deleterious free radicals, evoke mitochondrial injury and induce the release of inflammatory mediators, eventually leading to cell death. For instance, internal Ca^{2+} accumulation can result in the digestion of cellular proteins, lipids, nucleic acid by activating a variety of intracellular hydrolytic enzymes including calpains, nucleases and lipases. The Ca^{2+} -activated proteases are capable of degrading structural proteins, such as neurofilament, spectrin and myelin basic proteins, and functional proteins, such as ATPases, superoxide dismutase (Croall and DeMartino, 1991; Zimmerman and Schlaepfer, 1984), which are essential for cell survival.

1.1.2 Free radicals

More than 90% of oxygen that enters human cells is used for production of energy by mitochondrial cytochrome oxidase, and approximately 1-4% of absorbed oxygen forms reduced O_2 species. Acceptance of a single electron by O_2 generates a superoxide anion radical ($O_2^{\cdot-}$), which quickly undergoes dismutation to generate hydrogen peroxide (H_2O_2) by an important enzyme superoxide dismutase (SOD). In presence of transition metals, such as Fe^{2+} or Cu^{2+} , H_2O_2 is reduced to hydroxyl radical ($OH\cdot$) (Fenton reaction) (Bielski, 1991), which is highly destructive to cells by reacting rapidly with other molecules, and subsequently damaging nuclear and mitochondrial DNA, membrane lipids and carbohydrates. Under physiological conditions, the free radical generation is almost balanced by a variety of antioxidants including enzymatic (SOD), hydrophilic (ascorbate, urate, glutathione) and lipophilic (tocopherols, flavonoids) radical scavengers. Under certain conditions, such as trauma and ischemia, ion disruptions, particularly $[Ca^{2+}]_i$ elevation, may increase free radical production (Halliwell and Gutteridge, 1984; Ikeda and Long, 1990), and induce excessive $OH\cdot$ generation (Orrenius et al., 1989) through a variety of mechanisms (for review see Lipton, 1999), such as disturbed mitochondrial function and accumulation of the sources of free radicals including hypoxanthine (substrate for xanthine oxidase) and arachidonic acid (product of upregulated cyclooxygenase-2), eventually resulting in cellular damage when the role of free radicals overwhelms the antioxidant defense systems. Also, the destructive effects of radicals can result from other free radicals, particularly peroxynitrite anion ($ONOO^-$) generated by the interaction of $O_2^{\cdot-}$ with nitric oxide (NO), a product regulated by NO synthase activated by the Ca^{2+} /calmodulin system. The toxicity of $ONOO^-$ derives from

its ability to directly nitrate and hydroxylate the aromatic rings of amino acid residues, to react with sulphhydryls, with zinc-thiolate moieties, as well as with lipids, proteins and DNA, thus producing devastating effects on cells (Reiter, 1998). In addition, activation of poly (ADP-ribose) polymerase (PARP) by broken single-stranded DNA is able to deplete nicotinamide adenine dinucleotide (NAD) by cleaving this substrate (Szabo et al., 1996), further inhibiting mitochondrial function and ATP production.

The harmful effects of free radicals to living tissues indicate that this oxidative injury mechanism may be part of anoxic/ischemic and traumatic cell damage of white matter tracts, given that excessive radicals are generated during the pathological processes. There is evidence showing the direct link between ion deregulation and the formation of reactive oxidants. Experiments by Dykens et al (Dykens, 1994) demonstrate that a combination of increased intracellular Ca^{2+} and Na^+ with oxygen available for mitochondrial respiration can promote the generation of large amounts of reactive oxygen species (ROS) including hydroxyl radicals. Elevated $[\text{Ca}^{2+}]_i$ and subsequent mitochondrial Ca^{2+} accumulation can stimulate the production of superoxide and hydrogen peroxide by activation of xanthine oxidase (Dykens, 1994; Kinuta et al., 1989; Patt et al., 1988). Thus, following ischemia or trauma of myelinated axons, the collapses of ion homeostasis, such as Ca^{2+} overload, might induce excessive generation of free radicals in neural cells. Subsequently, these radicals may contribute to cellular damage through peroxidation of membrane lipids with release of harmful arachidonic acid, inhibition of various proteins including ion channels, transporters and enzymes involving energy metabolism and the damage to nuclear DNA (Hall, 1996). Notably, free radical

generation may also cause severe irreversible damage to Na⁺-K⁺-ATPase and make it more susceptible to proteolytic disruption (Chen et al., 1992; Huang et al., 1992). A recent report directly shows that a combination of internal Na⁺ load and excessive oxidative stress could induce ATP depletion and a progressive deregulation of intracellular Ca²⁺ and Na⁺ homeostasis (Chinopoulos et al., 2000). Thus, it is likely that there is a positive feedback among ROS formation, failure of energy metabolism and ion impairments during cell injury, which may further contribute to cell injury.

Recently, a variety of studies have provided substantial evidence for an involvement of free radicals in the pathophysiology of axonal injury. First, the studies with microdialysis indicate that the levels of ROS, such as hydrogen peroxide, are significantly elevated for a long period (~10hrs) after traumatic SCI (Liu et al., 1999a) and this alteration can be inhibited by administration of the antioxidant enzyme SOD (Liu et al., 1998). Second, elevation of oxygen species, such as NO and superoxide anion radical, may lead to remarkable axonal degeneration (Liu et al., 1999b; Lucius and Sievers, 1996), indicating the sensitivity of white matter tissues to free radical exposure. Finally, oxygen radical scavengers or antioxidants inhibiting free radical generation and reactivity have been shown to block posttraumatic pathophysiology and promote functional recovery and survival in experimental studies (Hall, 1989; Hall et al., 1992; Povlishock and Kontos, 1992). For instance, bifemelane, which has been shown to protect against cytotoxicity of hydrogen peroxide (Miyazaki et al., 1999), can reduce cell damage in ischemic CNS myelinated axonal injury in optic nerve by increasing the number of surviving axons (Adachi et al., 1996). Treatment with free radical scavengers, such as alpha-phenyl-N-

tert-butyl nitron (PBN) or 21-aminosteroid, could attenuate lactic acidosis and tissue damage, and improve energy metabolism and functional recovery after traumatic or ischemic SCI (Farooque et al., 1997; Francel et al., 1993), although the direct role of these drugs in gray matter could not be ruled out. The glucocorticoid steroid methylprednisolone, which possesses significant antioxidant efficacy (Hall, 1992), could improve chronic neurological recovery of patients or experimental animals after SCI via a mechanism independent of the steroid's glucocorticoid receptors (Hall et al., 1992). Taken together, free radicals appear to contribute to axonal dysfunction during white matter injury, and block of this aberrant pathway may be an efficient therapeutic target for acute pharmacological neuroprotection.

1.1.3 Mitochondria

Mitochondria play an important role in the survival of neural cells by involving a variety of important functions including generation of energy (ATP synthesis), ROS (such as superoxide radicals) (Turrens, 1997), regulation of intracellular Ca^{2+} by sequestering or releasing Ca^{2+} (Babcock and Hille, 1998), and modulation of Ca^{2+} -dependent permeabilization of their inner membrane (mitochondrial permeability transition, MPT) (Kroemer et al., 1998; Kroemer and Reed, 2000), a channel permeable to molecules and ions < 1.5 kDa (Crompton and Andreeva, 1993). Maintenance of adequate mitochondrial membrane potential (about 150 mV), generated principally by transmembrane proton gradients, is essential for performing these functions. Collapse of this potential such as by excessive Ca^{2+} accumulation into the matrix (Nicholls and Budd, 2000), would reverse ATP synthase, leading to rapid hydrolysis of cytoplasmic ATP because generation of

ATP requires hyperpolarized mitochondrial potential (Nicholls and Ward, 2000). For a cell to survive, the capacity of ATP generation by glycolysis must reach an essential level which exceeds that required by maintaining normal cell activity plus that consumed by reversed ATP synthase. During various pathological conditions such as ischemia or trauma, the fall of energy supply, together with $[Ca^{2+}]_i$ accumulation, would result in Ca^{2+} accumulation by mitochondria, which depolarizes mitochondrial membrane potential and leads to the impairment of mitochondrial functions (Nicholls and Ward, 2000), including failure of ATP generation, Ca^{2+} sequestration, and inadequate amounts of free radical generation, eventually causing the deleterious effects on the cell through necrotic mechanisms (Lipton, 1999; Nicholls and Budd, 2000; Siesjo et al., 1999). Moreover, mitochondria play a key role in the induction of cell death via apoptosis, usually initiated by Ca^{2+} efflux from this organelle (Chakraborti et al., 1999), through release of the apoptogenic factors cytochrome c and apoptosis-inducing factor (AIF) through the outer membrane (Green and Reed, 1998; Kroemer et al., 1998; Kroemer and Reed, 2000). The release of these factors, regulated by pro-apoptotic proteins such as Bax and anti-apoptotic proteins such as Bcl-2, could induce nuclear apoptosis by activating caspase3 and other caspases in the cytosol (Green and Reed, 1998; Kroemer et al., 1998; Thornberry and Lazebnik, 1998).

Ca^{2+} accumulation by mitochondria is a major mechanism of damage to this organelle and subsequent cell toxicity (Lipton, 1999; Stout et al., 1998), indicating that during the pathological process of anoxia/trauma, mitochondria may contribute to axonal injury when the elevated cytoplasmic free Ca^{2+} rises beyond a threshold value (set point), at

which mitochondria become to accumulate this cation (Nicholls and Ward, 2000). Morphological studies have demonstrated the characteristic changes of mitochondria during anoxia or trauma of myelinated axons. Following 60 min of anoxia in rat optic nerve, significant mitochondrial swelling with loss of normal cristae was seen in axoplasm under electron microscopy (Waxman et al., 1992), which is possibly evoked by Ca^{2+} accumulation and excess free radical generation (Lipton, 1999). Axotomy of spinal cord axons in larval sea lamprey resulted in degenerative changes including disruption of mitochondria to form large vacuoles near the lesion site (McHale et al., 1995). These observations provide morphological evidence for the involvement of mitochondria in anoxic/traumatic axonal injury. More studies recently demonstrate the functional damage to axonal mitochondria during axonal injury. Buki et al. (2000) report that axonal damage may lead to the release of cytochrome c from impaired mitochondria, which results in significant adverse effects by activating proteases in axons. Treatment with cyclosporin A, a blocker for MPT, as well as for calcineurin (Kroemer and Reed, 2000), protected axons from axotomic injury of brain white matter (Buki et al., 1999; Okonkwo et al., 1999; Okonkwo and Povlishock, 1999), indicating the involvement of mitochondria in axonal dysfunction, similar to the role of mitochondria in neuronal damage following transient forebrain ischemia (Siesjo and Siesjo, 1996).

1.1.4 Apoptosis

Generally, there are two types of cell death, necrosis resulting from injury (usually involving inflammatory processes *in vivo*), and apoptosis (programmed cell death) occurring during development or under certain conditions, though a third distinct form of

cell death, autophagocytosis, involving formation of autophagosomes by fusion of lysosomes with subcellular elements, has also been suggested (Clarke, 1990; Lipton, 1999). Apoptosis displays several distinct characteristics including cell shrinkage, membrane blebbing, chromatin condensation, and DNA fragmentation, which contrasts with those of necrosis, such as the swelling and rupture of cells and organelles. The most unequivocal morphological features of apoptosis are the formation of smoothly contoured spherical or lunar-shaped masses of chromatin in the nucleus and the subsequent formation of apoptotic bodies, membrane-bound structures containing cytoplasm and dark chromatin. One of the biochemical hallmarks of apoptosis is the cleavage of double-stranded DNA into nucleosomal segments at internucleosomal linker regions, resulting in ladder appearance of multiple DNA fragments on agarose gel electrophoresis (Compton, 1992). Labeling of DNA *in situ* using terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) is one of the most commonly used histochemical approaches to identify apoptotic cell death, although TUNEL is only relatively selective for apoptosis since labeling of both nucleosomal and nonnucleosomal fragments (the latter may appear during necrosis) takes place. In addition to an increase in the ratio of proapoptotic (Bax) to the antiapoptotic (Bcl-2, Bcl-xL) proteins of Bcl family, the central component of apoptosis is a proteolytic system involving a family of proteases, the caspases (cysteine proteases), which participate in a cascade triggered in response to proapoptotic signals, resulting in cleavage of a set of proteins and the subsequent disassembly of cells (Lipton, 1999; Thornberry and Lazebnik, 1998). Apoptosis in the CNS was previously thought to be primarily limited to developmental regulation of cell number, but more investigations with advanced techniques reveal that

the apoptotic characteristics occur under various pathological conditions, such as stroke and trauma. Thus, currently, apoptosis is not only recognized as a normal feature in the development of the nervous system, but is also involved in some neural diseases.

Both global (Heron et al., 1993; Nitatori et al., 1995) and focal (Du et al., 1996; Linnik et al., 1993) ischemic brain cell loss has been shown in part to involve apoptosis. In cortical cell cultures deprived of oxygen and glucose, neurons die predominantly by excitotoxic necrosis (Goldberg and Choi, 1993), but the neurons undergo apoptosis if the excitotoxicity is blocked by the combined application of NMDA and AMPA/kainate receptor antagonists (Gwag et al., 1995). Apoptosis of neural cells likely also occurs after traumatic insults (Rink et al., 1995) and during the pathophysiological process of neurodegenerative diseases (Portera-Cailliau et al., 1995; Thompson, 1995). By observing the morphology of nuclear chromatin (staining with Hoechst 33342 or TUNEL), DNA laddering on gel electrophoresis, and other characteristics of tissue damage, several authors have reported that apoptosis contributes to spinal cord tissue damage after mild to moderate traumatic injury involving both neurons and glia. Although apoptosis is found in both gray and white matter at the lesion site of SCI within the first 24 hours, significant oligodendrocytic apoptosis occurs especially in white matter, even at sites distant to the injury (Crowe et al., 1997; Liu et al., 1997). These findings suggest that the apoptotic cell death of oligodendrocytes may contribute to the disturbances of axonal function by inducing myelin degeneration (Abe et al., 1999; Li et al., 1999; Shuman et al., 1997; Yong et al., 1998). Interestingly, most recent experiments indicate that axonal trauma evokes the release of cytochrome c from mitochondria and

the subsequent activation of the caspases in axons, which are spatially and temporally connected to Ca^{2+} -induced, calpain-mediated spectrin proteolysis (Buki et al., 2000). These data demonstrate the partial overlap of molecular injury mechanisms between apoptosis and necrosis (Wang, 2000) following axonal injury. Taken together, apoptosis has been recognized as a common feature of delayed cell death in ischemic and traumatic axonal injury, particularly for oligodendrocytes. The mechanisms for apoptosis following ischemia or trauma are probably attributed to withdrawal of trophic factors (Raff et al., 1993), the generation of cytokines (Louis et al., 1993) and free radicals (Jacobson, 1996; Ratan et al., 1994a; Ratan et al., 1994b) and downregulation of protein kinases (MacManus et al., 1997; Morioka et al., 1992), but whether cell death occurs via apoptosis or necrosis appears to depend on the severity and/or duration of the insults (Lee et al., 1999).

1.1.5 Glutamate toxicity

Excitotoxicity has been invoked as a major mechanism of anoxic/ischemic neuronal injury (Choi, 1992b; Olney and Sharpe, 1969). Under energy-limited conditions, depolarization of synaptic terminals causes excessive release of the excitatory neurotransmitter glutamate and such an uncontrolled glutamate efflux, coupled with impaired uptake mechanisms, leads to pathological overactivation of postsynaptic NMDA and AMPA/kainate receptors, which in turn culminates in neuronal death from Na^+ and Ca^{2+} overload. In contrast, astrocytes are far more resistant to oxygen deprivation than neurons and axons (Goldberg and Choi, 1993), though the metabolic rates of these cells in mammals are comparable (Hertz and Peng, 1992). However, combined anoxia

and aglycemia can greatly increase the impairment to this type of glial cell (Ransom and Fern, 1996). The oligodendrocyte and its precursor cell are extremely sensitive to anoxic/ischemic and traumatic insults (Corley and Yong, 1999; Husain and Juurlink, 1995; Jelinski et al., 1999). The mechanisms of glial injury from energy deprivation are not clear, possibly involving intracellular Ca^{2+} accumulation via L-type VGCC, ionotropic glutamate receptors, reversed Na^+ - Ca^{2+} exchange and even release from internal Ca^{2+} stores (Duffy and MacVicar, 1996; Holgado and Beauge, 1995; Holgado and Beauge, 1996; Takuma et al., 1994), together with peroxidation of membranes, cellular edema and intracellular acidosis (Ransom and Fern, 1996). Interestingly, during anoxic exposure, myelin regions accumulate more Ca^{2+} than other glial processes or somata (LoPachin and Stys, 1995), possibly indicating direct damage to this important structure under pathological conditions.

Excitotoxicity undoubtedly plays a major role in ischemic gray matter damage, but the mechanism of damage to another vital component, the white matter, is less well understood. Given the non-synaptic nature of white matter, one might dismiss excitotoxicity from playing any role in this tissue. A series of studies published in the early 1990's appeared to support the notion that glutamate-dependent mechanisms contribute little, if at all, to anoxic or ischemic white matter damage. For example, the NMDA receptor antagonist ketamine, when applied at concentrations that are specific for NMDA antagonism, fails to improve electrophysiological recovery in an *in vitro* model of white matter anoxia (Ransom et al., 1990). However, the possible role of ionotropic non-NMDA receptors, or even metabotropic glutamate receptors, had not been ruled out

by this work. A number of studies indicate the presence of non-NMDA receptors on astrocytes (Barres, 1991; Hosli and Hosli, 1993). PCR analysis shows the appearance of AMPA receptor subunits in developing and adult rat optic nerve, indicating the receptor expression by astrocytes, oligodendrocytes and/or O-2A progenitor cells (Jensen and Chiu, 1993). Later, several subunits of AMPA and kainate receptors were conclusively identified on astrocytes in the brain and spinal cord white matter (Agrawal and Fehlings, 1997; Garcia-Barcina and Matute, 1998), with differences in subunit complement apparently depending on which region was examined. When glutamate receptor activation is excessive (desensitization is pharmacologically blocked), astrocytes are injured by non-NMDA receptor stimulation (David et al., 1996). Oligodendroglia and their precursors have also been found to express AMPA and kainate selective receptors (Garcia-Barcina and Matute, 1996; Matute et al., 1997; Patneau et al., 1994; Yoshioka et al., 1995). Application of AMPA or kainate to oligodendrocytes either *in vitro* or *in situ* cause significant increases in cytosolic $[Ca^{2+}]$ and cell death. Consistent with this is the identification of the AMPA receptor subunits GluR3 and GluR4, and the kainate receptor subunits GluR5-7, KA1 and KA2 in oligodendrocytes, although some minor differences exist between cultured and cells *in situ* receptors (Garcia-Barcina and Matute, 1996; Matute et al., 1997). The lack of GluR2 subunits indicates that AMPA receptors in these cells are Ca^{2+} -permeable and may mediate Ca^{2+} -dependent cell death upon exposure to agonist. Thus, the above findings indicate that AMPA/kainate receptors may significantly contribute to white matter injury.

While excitotoxic mechanisms are well known in gray matter, and glial cells appear vulnerable to excitotoxins, only recently have studies elucidated the potential importance of glutamate-dependent injury in isolated white matter tracts. Traumatic SCI depends in part on excitotoxic mechanisms both *in vitro* (Agrawal and Fehlings, 1997) and *in vivo* (Rosenberg et al., 1999a; Wrathall et al., 1992). Pharmacological inhibition of AMPA/kainate receptors with NBQX or CNQX significantly improves outcome after experimental spinal cord trauma, with improvement in function (Agrawal and Fehlings, 1997) and ultrastructural appearance of glia and myelin sheaths (Rosenberg et al., 1999a). In addition, kynurenic acid, a broad spectrum glutamate receptor antagonist, significantly improves behavioral outcome in an *in vivo* rat model of spinal cord contusion (Wrathall et al., 1992), although it is uncertain whether these effects are directly related to white matter sparing or mediated indirectly through effects on gray matter or the vasculature. Taken together, enough evidence has accumulated to implicate excitotoxic mechanisms that target white matter elements directly, with glial cells being suspected victims.

1.2 Structure and function of AMPA receptors

Glutamate receptors mediate most excitatory neurotransmission in the CNS, and participate in plastic changes in the efficacy of synaptic transmission underlying learning, memory and formation of neural networks during CNS development. Glutamate receptors are categorized into two classes, ionotropic and metabotropic glutamate receptors. The ionotropic receptors, which include cation-specific channels, are divided into three groups: AMPA, kainate and NMDA receptors. The metabotropic receptors (mGluR) are coupled to GTP-binding proteins (G-protein) and modulate the production

of intracellular messengers by linking to activation of phospholipase C or inhibition or activation of adenylyl cyclase. In this section, the fundamental structure and function of the AMPA glutamate receptors are reviewed in detail because, as shown in *Chapter 2-4*, my studies have indicated a major role of this subtype of ionotropic receptor in the excitotoxicity of CNS myelinated axons.

Natural AMPA receptors are heteromeric complexes of four homologous subunits (GluR1-4) that combine differentially to form a variety of AMPA receptor subtypes. The subunits of AMPA receptors have a large extracellular amino-terminal domain, three transmembrane domains and an intracellular carboxy-terminal domain (Fig. 2). AMPA receptors can be found throughout the CNS, but are largely localized at excitatory synapses. When activated (opened) and desensitized (closed) by glutamate, AMPA receptors typically produce a brief excitatory postsynaptic potential (EPSP) due to rapid desensitization. AMPA receptors mediate the majority of fast excitatory neurotransmission in the synapses of CNS and play a key role in synaptic plasticity underlying learning and memory (Hollmann and Heinemann, 1994; Nicoll and Malenka, 1995).

1.2.1. Structure

Four AMPA glutamate subunits, GluR1, GluR2, GluR3 and GluR4 have been identified with expression cloning techniques. AMPA receptors, either homomeric or heteromeric oligomers composed of these multiple subunits, share a tetrameric structure with voltage-gated K⁺ channels (Roche et al., 1996; Rosenmund et al., 1998), though pentameric

structure has also been proposed. The remarkable differences in functional properties of native AMPA receptors are produced by different assemblies of these subunits. These AMPA receptor subunits have a similar size (~900 amino acids, ~100kDa) and share 68%-73% amino acid sequence identity (Hollmann and Heinemann, 1994; Seeburg, 1993). Each of the GluR1-4 subunits exists in two different isoforms, “flip” and “flop”, because of alternative splicing of exons encoding a 38 amino acid sequence preceding the M4 segment (Fig. 2) (Sommer et al., 1990). These two alternative forms of GluR subunits exhibit differential expression during development.

RNA editing increases the diversity of receptor subunits. An intronic site preceding the flip/flop site is subject to editing from arginine to glycine (R to G) and modifies the kinetic response to glutamate for recombinant channels. The receptors with glycine residues at this site result in a more rapid recovery from desensitization. This RNA editing may occur for GluR2, GluR3 and GluR4 but not for GluR1 (Lomeli et al., 1994). A glutamine residue (Q) in M2 is encoded in the gene for GluR1-4, but the GluR2 cDNA clones in adult contain arginine (R) at this position called Q/R (Sommer et al., 1991; Wisden and Seeburg, 1993). This codon alteration of adenosine(A)-to-guanosine(G) is generated by site-directed nuclear RNA editing, and only low levels of unedited RNA are present in fetal brain (Burnashev et al., 1992; Higuchi et al., 1993).

Immunocytochemical and biochemical experiments have indicated an intracellular location of the C-terminus (Tingley et al., 1993). In order to determine the transmembrane topology of the ionotropic glutamate receptors, some investigators have

developed a series of mutants of AMPA subunit GluR1 by introducing N-glycosylation consensus sequences at different sites along the entire protein, and then analyzed these mutant receptors for glycosylation, which can be considered a marker of extracellular localization of the respective sites (Hollmann and Heinemann, 1994). These investigations suggest that AMPA receptors have only three transmembrane domains, M1, M2 and M3. The previous proposed transmembrane M2 does not span the membrane, but is only close to the intracellular surface of plasma membrane or makes a re-entrant loop (hairpin turn) within the membrane (Fig. 2). Although the C-terminus is intracellular, the entire region between M3 and M4 is localized to extracellular part. This model is not only applicable to AMPA receptor subunits, but also to kainate and NMDA receptor subunits.

1.2.2. Functional properties

1.2.2.1 Ca^{2+} permeability and rectification of AMPA receptors

The Ca^{2+} permeability of a channel is usually determined by the relative Ca^{2+} to monovalent ion permeability ratio calculated from reversal potentials using the Goldman-Hodgkin-Katz equation, although this ratio does not show how much Ca^{2+} enters the cell during activation of ligand-gated channels. The fractional Ca^{2+} current (Pf, a ratio of charge carried by Ca^{2+} to the total charge carried by all permeant ions) is used to quantify the Ca^{2+} flux. Pf can be obtained by calculation of relative Ca^{2+} to monovalent ion permeability or by simultaneous measurements of whole-cell current and fluorescence signal of Ca^{2+} -sensitive dye (Burnashev, 1996). Permeability studies have shown that AMPA receptors have a substantial permeability to Ca^{2+} and a strong inward rectification

in a small population of cultured rat hippocampal neurons (type II), but they display a linear or slight outward rectification and little permeability to Ca^{2+} in most neurons (type I) (Iino et al., 1990; Ozawa and Iino, 1993). Glial cells in both gray and white matter have also been shown to express AMPA receptors with a variable Ca^{2+} permeability (Berger, 1995; Burnashev et al., 1992; Seifert and Steinhauser, 1995).

1.2.2.2 Role of GluR2 in determining Ca^{2+} permeability and rectification

Studies from GluR1-4 expression using *Xenopus* oocytes or human embryonic kidney (HEK 293) indicate that the functional properties of recombinant AMPA receptors depend on their subunit compositions. Homomeric receptors assembled from GluR2 subunits have little Ca^{2+} permeability and an outward rectification. In contrast, homomeric receptors assembled from GluR1, GluR3 and GluR4 subunits have high Ca^{2+} permeability and a strong inward rectification. In the heteromeric AMPA receptors, GluR2 subunit is very important in determining both Ca^{2+} permeability and rectification properties. The recombinant receptors coexpressing GluR2 with GluR1, GluR3 and/or GluR4 have little Ca^{2+} permeability and an outward rectification (Hollmann and Heinemann, 1994; Jonas and Burnashev, 1995; Jonas et al., 1994). The AMPA receptor mediating Ca^{2+} influx in neurons is only regulated by the levels of Q/R site edited GluR2, not by the unedited GluR2. A targeted GluR2 gene mutant with 30% reduced GluR2 levels has 2-fold higher Ca^{2+} permeability in pyramidal cells (Kask et al., 1998). Thus, the relative abundance of GluR2 mRNA is negatively correlated with Ca^{2+} permeability of AMPA receptors, and therefore is the main determinant of Ca^{2+} permeability (Bochet et al., 1994; Kondo et al., 1997).

1.2.2.3 Role of Q/R site in modulating Ca^{2+} permeability

The mechanism of GluR2-mediated Ca^{2+} permeability is related to a critical arginine (R586) residue in M2 loop of this subunit since this positively charged residue hinders the permeation of divalent cations including Ca^{2+} . In contrast, in the other subunits, a homologous amino acid glutamine with a neutral charge is present at the same site (Q/R site). The mutant GluR2(Q) displays similar Ca^{2+} permeability and rectification to wild-type GluR1, GluR3 and GluR4 when the arginine in this site of GluR2 is substituted by glutamine through site-directed mutagenesis. Thus, the presence of GluR2, which has an arginine residue in the hairpin loop at the internal mouth of the channel, prevents the permeation of Ca^{2+} and other divalent cations, because the arginine in the pore-lining segments forms a positively charged ring close to the narrow portion of the channel and constitutes a energy barrier for entry of cations including Ca^{2+} . In heteromeric channels, the ring contains both arginine and glutamine, the reduced positively charged arginines decrease the entry resistance for divalent cations. In homomeric Q-form channels, this resistance is further lowered and results in a larger inflow of Ca^{2+} (Burnashev, 1996). Since the arginine in the Q/R site is regulated by RNA editing via replacing gene-coded glutamine, Ca^{2+} entry into neurons through AMPA receptors is prevented by RNA editing in adult brain. Thus, editing-created codon changes in AMPA receptor subunits affect amino acid positions in M2 (forming pore lining segment), and alters the biophysical properties of AMPA receptors (Seeburg et al., 1998). Expression of specific subunits as well as the editing of pre-mRNA is an important means of regulating AMPA receptor structure and function.

1.2.2.4 Rectification of AMPA receptors

The inward rectification property of Ca^{2+} -permeable AMPA receptors is mediated by physiological concentrations of polyamines (e.g. spermine and spermidine) (Donevan and Rogawski, 1995). Removing naturally occurring polyamines from inside of a test cell or excised patch results in loss of inward rectification without affecting Ca^{2+} permeability in Ca^{2+} -permeable AMPA receptors. At moderately positive potentials, the intracellular polyamines enter the channel pore and block the ion flow when the Q/R site is occupied by glutamine. At more positive potentials, the polyamines may permeate through the channel and ion flow is restored. In channels containing subunits with arginine in the Q/R site, the electrostatic interaction between the positively charged arginine and polyamines prevents the entry of polyamines into the channel pore. Thus, native and recombinant AMPA receptors with high Ca^{2+} permeability are about 1000-fold more sensitive to polyamines than those with low Ca^{2+} permeability (Koh et al., 1995). Mutant channels containing an asparagine at the Q/R site, though highly Ca^{2+} permeable, show a linear I-V relationship as they are insensitive to polyamines (Dingledine et al., 1992).

1.2.2.5 Physiological roles of Ca^{2+} -permeability

In most CNS neurons, native AMPA receptors, heteromers of GluR1-GluR4, are poorly permeable to Ca^{2+} since GluR2 is ubiquitously expressed in CNS. However, some neural cells, such as type II cultured hippocampal neurons, cerebellar Bergmann glia, hippocampal and neocortical interneurons, possess native AMPA receptors with a relatively high Ca^{2+} permeability (Iino et al., 1990; Isa et al., 1996; Itazawa et al., 1997).

The Ca^{2+} -permeable AMPA receptors contribute to excitatory synaptic transmission in hippocampal, neocortical, nonpyramidal and spinal dorsal horn neurons (Gu et al., 1996; Isa et al., 1996; Itazawa et al., 1997), and provide a synaptically activated route for Ca^{2+} entry, involving the regulation of long-term synaptic function. For instance, in GluR2 mutant mice, the Ca^{2+} permeability of AMPA receptors in CA1 pyramidal cells increases significantly (~9 times), and at the same time, long-term potentiation (LTP) in CA1 synapses is remarkably enhanced. In CA1 of hippocampus, LTP is initiated by transient NMDA receptor activation, and then persists during synaptic transmission via AMPA receptors by postsynaptic modulation of AMPA receptor function through an increase in single channel conductance of AMPA receptors (Benke et al., 1998), implicating postsynaptic Ca^{2+} entry through the colocalized AMPA and NMDA receptors as a fundamental mechanism for excitatory synaptic transmission (Jonas and Burnashev, 1995). Thus, Ca^{2+} entry via the Ca^{2+} -permeable receptors can regulate synaptic plasticity under certain conditions (Jia et al., 1996; Staubli and Chun, 1996), alterations in the properties of synaptic AMPA receptors probably function as one of the plausible mechanisms for modifying synaptic efficacy underlying learning and memory.

1.2.3 Kinetics

AMPA, glutamate and kainate are representative agonists of AMPA receptors. Except for kainate, AMPA and glutamate induce rapidly and profoundly desensitizing responses, a major kinetic property of AMPA receptors. When activated by AMPA or glutamate, these receptors enter a liganded but closed (desensitized) state with a characteristic time course (Waters and Allen, 1998). The kinetics of native and recombinant AMPA receptor

desensitization has been studied in outside-out membrane patches by rapidly applying agonists (Geiger et al., 1995; Mosbacher et al., 1994). The time constants of desensitization of current responses are variable from 1-16ms (~5ms in most cases) in outside-out patches induced by 1-10ms glutamate. This large variation in desensitization kinetics probably arises from different subunit composition of the AMPA receptors tested because different recombinant homo- and heteromeric AMPA receptors have variable desensitization properties. Among homomeric GluR1, GluR3 and GluR4 receptors, the GluR4 flop channel has the fastest desensitization time constant (0.9ms), and GluR3 flip the slowest (4.8ms) when 1 mM glutamate is applied (Mosbacher et al., 1994). Among heteromeric AMPA receptors assembled from flop or flip forms of GluR2 and GluR4 subunits, the GluR2 flop/GluR4 flop channel has the fastest time constant (0.8ms), while GluR2 flip/GluR4 flip has the slowest (6.1ms), indicating that channels assembled from flop forms display faster desensitization than those with flip forms except for GluR1 subunit (Ozawa et al., 1998). Therefore, alternative splicing of AMPA receptor subunits regulates the channel kinetics, and may affect synaptic currents. Furthermore, RNA editing at the R/G site also regulates desensitization kinetics. AMPA receptors assembled from edited forms have a slower desensitization than those from unedited forms. Most recent research shows that molecular determinants of desensitization are localized to a small region (38 amino acids) in flip/flop domain of S2 segment, especially the residue 750 (Partin et al., 1996), although three residues (T504, L507 and R509) in S1 of GluR3 have also been shown to modify AMPA receptor desensitization, with a major role for L507, which forms the structure controlling agonist binding (Stern-Bach et al., 1998).

In CNS synapses, AMPA receptors are desensitized if glutamate is present in the synaptic cleft at an adequate concentration for a period longer than the intrinsic time course of desensitization. The rapid desensitization of AMPA receptors plays a physiological role in protection of cells from overactivity and in the regulation of synaptic responses (Funk et al., 1995; Yamada and Rothman, 1992). For example, these rapid AMPA receptors may be useful in transmitting signals necessary for sound localization (Ballerini et al., 1995; Raman et al., 1994), and desensitization may contribute to synaptic depression and prevent the over-interaction of transmitter quanta within the synaptic cleft (Otis et al., 1996; Trussell et al., 1993).

1.2.4 Single channel properties

Recent studies have investigated the effects of RNA editing at Q/R site and splice variants of flip and flop on the single channel properties of recombinant AMPA receptors of GluR2 and GluR4 subunits (Swanson et al., 1997). The single channel conductance is highest (8-24 pS) for the Ca^{2+} -permeable heteromeric AMPA receptors (unedited GluR2 flop/GluR4 flip), lowest (~300 fS) for Ca^{2+} -impermeable homomeric AMPA receptors assembled entirely from edited GluR2 flip or flop, and intermediate (4-10 pS) for Ca^{2+} -impermeable heteromeric channels with both edited and unedited subunits (GluR2 flip/GluR4 flip and GluR2 flop/GluR4 flip). Therefore, the single channel conductance of AMPA receptor is dependent on the Q/R site editing state of the subunits comprising the channel, and is regulated by the expression of edited GluR2 subunits in neurons (Swanson et al., 1997).

1.2.5 Binding sites

AMPA receptors possess at least three separate binding sites for agonists, antagonists or desensitization modulators.

1.2.5.1 Binding sites for agonists and competitive antagonists

For a given AMPA receptor, the average conductance of its channel depends on how many subunits are occupied by the agonist (Rosenmund et al., 1998). Binding of two agonist molecules is required to activate AMPA receptor, but the two binding sites are neither identical nor independent (Clements et al., 1998). Quinoxalinediones, such as NBQX, the competitive AMPA receptor antagonists, also bind to these sites, but do not open the ion channel. The binding sites for the above agents have been investigated by exchanging portions of GluR3 and GluR6 (kainate receptor) subunits. Two extracellular discontinuous segments of ~150 amino acids residues, S1 (between N-terminus and M1) and S2 (between M3 and M4), determine the binding properties of these agents (Chen and Gouaux, 1997; Stern-Bach et al., 1994). In particular, amino acid residues 372-395 are important as an agonist receptor in the GluR3 subunit (Carlson et al., 1997). Analogous to the structure of lysine-arginine-ornithine-binding protein (LAOBP), S1 and S2 form two lobes that constitute an amino acid binding pocket. The flip/flop segment of the AMPA receptor might couple the movement of the ligand-binding lobes to channel regions since this segment modulates the extent of opening, closure and pivot movements of lobes. Consistent with this notion is the three transmembrane topology model of ionotropic glutamate receptors and the finding from mutant studies, which show that

mutagenesis of a number of residues of S1 of GluR1 alters the responses to agonist (Uchino et al., 1992).

1.2.5.2 Binding sites for positive and negative allosteric modulators

Several classes of drugs can positively modulate AMPA receptor activity: pyrrolidones (aniracetam, piracetam), benzothiadiazides (cyclothiazide, diazoxide) and sulfonylamino compound (Arai et al., 1996; Bleakman and Lodge, 1998; Sekiguchi et al., 1997). These drugs act on the AMPA receptors and slow the desensitization rate and/or deactivation and increase the affinity of sensitized states for agonist (Arai et al., 1996; Funk et al., 1995). The site of cyclothiazide (CTZ) action has been localized to the flip and flop splice variants of homomeric GluR1 AMPA receptors. The receptors assembled from flop forms are more affected by CTZ than those of flip forms. There are some amino acid differences between flip and flop forms in three separate areas (Fig. 2), but a single amino acid site at position 750 is most sensitive to CTZ because exchange of serine-750 (flip) and glutamine-750 (flop) shows reversed effects on CTZ sensitivity (Partin et al., 1995; Partin et al., 1996). Aniracetam binds at or near the same critical site in flip/flop region as CTZ, but acts by a different mechanism. Aniracetam causes desensitization or deactivation by reducing the closing rate constant for ion channel gating, while CTZ produces action by stabilizing a nondesensitized agonist-bound closed state (Partin et al., 1996). In contrast, 2, 3-benzodiazepines, such as 1-(4-aminophenyl)-4-methyl-7, 8-methylenedioxy-5H-2, 3-benzodiazepine (GYKI52466) and 1-(4-aminophenyl)-4-methyl-7, 8-methylenedioxy-5H-2, (3N-methylcarbamate)-2, 3-benzodiazepine (GYKI53655), the highly selective non-competitive AMPA receptor antagonists

(Bleakman and Lodge, 1998; Donevan and Rogawski, 1993), produce blocking effects by increasing the rate of desensitization of AMPA receptors via binding to the CTZ-binding site or a neighboring site (Partin et al., 1996).

1.2.5.3 Binding sites for ion channel blockers

Several toxins, such as Joro spider toxin (JSTX), argitoxin and philanthotoxin, have been shown to block AMPA excitatory transmission. JSTX, with a polyamine moiety connected to a phenyl ring, has 2-3 positive charges at physiological pH. It blocks inward rectification and Ca^{2+} -permeability of AMPA receptors lacking GluR2 subunits (Iino et al., 1996), but does not affect outwardly rectifying Ca^{2+} -impermeable AMPA receptors formed with GluR2. The effects of JSTX are use-dependent and highly voltage-dependent since at positive potentials it has no blocking effect. The binding site for JSTX is near the central pore region of the channel (Blaschke et al., 1993), where the positive charges play a key role in the blocking effect. In addition, the edited GluR2 subunit also regulates the channel block sensitivity by external polyamines (e.g. argitoxin) or internal blocker (Herlitze et al., 1993; Magazanik et al., 1997; Washburn et al., 1997).

1.3 Properties of Na^{+} -dependent glutamate transporter

One of the key issues during glutamate transmission is removal of glutamate from the synaptic cleft, partly achieved by a powerful transport system, which has a high affinity for glutamate and exhibits a specific coupling to inorganic ions. The high affinity glutamate transporters, present on the presynaptic membrane or glial cells surrounding synapses, play an important role in termination of glutamatergic activity (Conradt and

Stoffel, 1995). Presynaptic glutamate transporters sequester glutamate directly from the synaptic cleft, whereas transporters on the surrounding glial cells maintain a low level of extracellular glutamate by forming a diffusion gradient favoring movement of glutamate away from the synapse. Generally, uptake of glutamate into glia, particularly the astrocytes, is greater than that into glutamatergic and other types of neurons (Drejer et al., 1983; Rothstein et al., 1996). So far, several types of glutamate transporter have been identified in mammals, including those localized to plasma membrane and subcellular organelles (such as proton-dependent mitochondrial glutamate symporter and antiporter). Out of several plasma membrane glutamate transporters, only the Na^+ -dependent glutamate transporter (using Na^+ , K^+ electrochemical gradient as a driving force), is coupled to an ionic gradient, permitting transport of glutamate against its concentration gradient. To date, five different members of Na^+ -dependent glutamate transporters (GLAST/EAAT1, GLT1/EAAT2, EAAC1/EAAT3, EAAT4 and EAAT5) have been cloned, and many molecular and functional properties of these transporters, particularly the first three isoforms, have been investigated recently. In the present study, I hypothesize that the operation mode of Na^+ -dependent glutamate transporters is reversed under pathophysiological conditions such as anoxia and trauma, leading to uncontrolled efflux of this amino acid (see 1.4).

1.3.1 Cloning and Structure

Danbolt et al. (1990) purified glutamate transporter 1 (GLT1) glycoprotein (73 kDa) and developed the antibody against this protein (Danbolt et al., 1990), which was used to isolate a cDNA clone and express this protein in Hela cells (Pines et al., 1992). The clone

of excitatory amino acid carrier 1 (EAAC1) was isolated from rabbit intestine and expressed in *Xenopus* oocytes (Kanai and Hediger, 1992). The glutamate aspartate transporter (GLAST, 66 kDa hydrophobic glycoprotein) was cloned and expressed in *Xenopus* oocytes (Storck et al., 1992). Later, human homologs of these three transporters were isolated (Arriza et al., 1994; Kanai et al., 1994; Manfras et al., 1994; Shashidharan and Plaitakis, 1993; Shashidharan et al., 1994), and called excitatory amino acid transporter 1-3(EAAT1-3, i.e., GLAST, GLT1 and EAAC1, respectively). Recently, another two isoforms of high affinity Na⁺-dependent glutamate transporters, human EAAT4 and EAAT5, have also been cloned (Arriza et al., 1997; Fairman et al., 1995).

Studies of hydropathy profiles and membrane topology suggest that glutamate transporters have a global structure that is unique among secondary transporters. Hydropathy plots reveal that several well-conserved segments form transmembrane α -helices (Gegelashvili and Schousboe, 1997). Although the exact number of membrane-spanning domains is still debatable, it is likely that glutamate transporter possesses eight membrane-spanning α -helices and a loop-pore structure, which is unique among secondary transporters. Another distinctive structural feature is the presence of a highly amphipathic membrane-spanning helix, which provides a hydrophilic path through membrane. An important region, which contains approximately 150 residues in the C-terminal of the proteins, forms four well-conserved sequence motifs, all of which have been suggested to be part of translocation pore or substrate binding site (Slotboom et al., 1999).

1.3.2 Tissue distribution and cellular localization

1.3.2.1 Tissue distribution

EAAC1 is abundant in brain though it was first cloned from rabbit intestine and was expressed outside the nervous system including kidney, heart, lung, muscle, placenta and liver (Gegelashvili and Schousboe, 1998; Robinson and Dowd, 1997). In contrast, GLT1 and GLAST are practically nervous system-specific proteins in spite of the existence of low density mRNA in other tissues (Rothstein et al., 1994). EAAT4 mRNA is mainly confined to cerebellum, while a low level of transcript is present in placenta (Fairman et al., 1995). Thus, within the nervous system, EAAC1, GLAST and GLT1 could be found throughout brain and spinal cord, whereas EAAT4 and EAAT5 seem to be strictly confined to cerebellum and retina, respectively. Cerebellum is specifically enriched in GLAST, but GLT1 is more abundant in cerebral structures, particularly telencephalic region. EAAC1 has a relatively even distribution among different brain regions with high levels in cerebral cortex (Nakayama et al., 1996; Rothstein et al., 1994).

1.3.2.2 Neuronal localization

EAAC1, a major transporter in neurons, is robustly expressed in glutamatergic and non-glutamatergic neuronal cells (Rothstein et al., 1994; Torp et al., 1994), and principally restricted to the dendrosomatic compartment, although some immunostaining was detected in presynaptic boutons of GABAergic neurons (Rothstein et al., 1994). The functional significance for the dendritic and somatic glutamate transporter is not understood, but the presence of transporter on presynaptic site is consistent with the conventional notion of terminating the synaptic action of glutamate and recycling

glutamate to synaptic vesicles for re-use as a transmitter. GLAST and GLT1, seemingly glial transporters, are expressed in neuronal cells as well. For example, some cerebellar neurons, such as non-glutamatergic cells, express GLAST (Rothstein et al., 1994). GLAST mRNA is found in rat cochlear hair cells (neurons) (Li et al., 1994) and in PC12 neuronal cells (Ramachandran et al., 1993). GLT1 is detected in different type of retinal bipolar cells (Neurons) in rats and primates (Euler and Wassle, 1995; Grunert et al., 1994; Rauen and Kanner, 1994), its mRNA is found in some hippocampal neurons (Torp et al., 1994). EAAT4 is expressed postsynaptically in cerebellar Purkinje cell dendrites (Yamada et al., 1996). EAAT5 is cloned from retina (Arriza et al., 1997). Both EAAT4 and EAAT5 proteins are highly coupled to Cl⁻ conductance (Arriza et al., 1997; Fairman et al., 1995), though its significance is unclear.

1.3.2.3 Glial localization

GLT1 and GLAST are expressed in astrocytes of brain, spinal cord and retina (Danbolt et al., 1992; Lehre et al., 1995; Levy et al., 1993), and both types of transporters could be co-expressed in the same astrocytes (Chaudhry et al., 1995; Lehre et al., 1995). Subcellularly, astroglial GLAST and GLT1 have been localized to the plasma membrane with higher density in thin processes (Chaudhry et al., 1995; Danbolt et al., 1992; Lehre et al., 1995; Rothstein et al., 1994), and co-localized with functionally-coupled glutamine synthetase, an enzyme that converts glutamate to glutamine (Derouiche and Rauen, 1995). However, GLT1 and GLAST proteins are not expressed uniformly in the membrane of astrocytes. The membrane facing nerve terminals, axons and dendritic spines displays higher density than that facing other glial cells, capillaries or pia

(Chaudhry et al., 1995). The astroglial processes anatomically close to glutamatergic or GABAergic synapses express higher level of glutamate transporters than the other part of surface. In contrast, GLAST immunoreactivity detected in neurons is specifically confined to the intracellular compartment, Golgi apparatus (Rothstein et al., 1994). These distributions are congruent with the major role of glial cells in taking up glutamate and maintaining low levels of this toxin around neural cells. In addition, GLAST and EAAC1 mRNA and proteins are found in oligodendrocyte, though it seems that this cell type does not express GLT1 transporter (Domercq and Matute, 1999; Domercq et al., 1999; Kondo et al., 1995; Kugler and Schmitt, 1999).

1.3.3 Kinetic and functional properties

The electrogenic properties of glutamate uptake has been established well before the glutamate transporter was successfully cloned (Kanner and Sharon, 1978; Schousboe et al., 1977). Extracellular Na^+ is absolutely required for the transport activity, which is also dependent on internal K^+ (or substitute ions such as Rb^+ or Cs^+). Glutamate uptake by transporters EAAT1-4 is almost abolished by removal of extracellular Na^+ , suggesting the co-transport of Na^+ with glutamate. A transport model suggests that a sequential binding or simultaneous translocation of 3 Na^+ , one proton, and one negatively charged glutamate ion followed by a counter-transport of one K^+ to exterior. Another potential mechanism utilizes co-transport of 2 Na^+ , one glutamate, and efflux of one K^+ and one OH^- (or HCO_3^-). Thus, there is inward movement of a net positive charge in either model (Gegelashvili and Schousboe, 1997; Zerangue and Kavanaugh, 1996). Transporting 2-3 Na^+ and 1 glutamate anion into the cell, and 1 K^+ out of the cell, glutamate transporters also

generate pH changes, acidic inside and alkaline outside the cell (Bouvier et al., 1992; Nelson et al., 1983). The alterations in pH are produced by transport either of 1 H⁺ into the cell or of 1 OH⁻ out of the cell with each glutamate anion, though the function of H⁺ or OH⁻ transport is unclear. Moreover, extra charges may be produced by Cl⁻ efflux through glutamate transporter, although the glutamate transport is not powered by Cl⁻ gradients (Billups et al., 1996). The Cl⁻ flux through glutamate transporter is different for various transporter subtypes: approximately 1 Cl⁻ per glutamate transport for EAAT1-3 (Wadiche et al., 1995a; Wadiche et al., 1995b), but much more Cl⁻ flux occurring for EAAT4 (Fairman et al., 1995).

The kinetic parameters of various glutamate transporters have been established in different brain regions and cellular preparations. For example, the affinities for glutamate/aspartate are high for rabbit EAAC1 (K_m 12 μM), rat GLT1 (K_m 2 μM) and human EAAT4 (K_m 1.0-2.5 μM), but low for rat GLAST (K_m 77 μM) (Fairman et al., 1995; Kanai and Hediger, 1992; Pines et al., 1992; Storck et al., 1992), indicating the functional discrepancies for the transporters GLAST, GLT-1 and EAAC1. GLT1, with a K_m of 2 μM for L-glutamate, is suggested to play an essential role in maintaining extracellular glutamate concentration at a low level. EAAC1, with a K_m of 12 μM for L-glutamate, is proposed to be the presynaptic glutamate uptake carrier. In contrast, GLAST, mainly localized in glial cells and having a high K_m for glutamate, may function as a reserve transporter to protect neurons from toxic glutamate levels. However, the above suggestions need to be confirmed since there is report showing that human homologues of GLAST, GLT1 and EAAC1 have similar affinities to L-glutamate and D-

or L-aspartate (Arriza et al., 1994). It is worth noting that the turnover rate of glutamate transporter is usually slow (70 ms/cycle, or 14 cycles/sec) (Wadiche et al., 1995b). To keep external glutamate at low levels, cells must therefore express high densities of transporters, which appear to be between 1500-1800/ μm^2 (Dehnes et al., 1998; Vandenberg, 1998).

Recently, using molecular biological methods, Rothstein et al. (1996) showed that removing glial transporters elicits a general rise of extracellular glutamate concentration, subsequently causing cell death. Inhibition of EAAC1 expression induces epileptic-like seizures, although it does not raise the levels of this excitatory amino acid. These findings suggest that glial transporters function mainly to keep the glutamate concentration low in the extracellular space, whereas neuronal transporters (EAAC1) play a more specific role in synaptic transmission.

1.4 Hypotheses and objectives

Recent reports using *in vitro* and *in vivo* models suggest that excitotoxic mechanisms involving non-NMDA receptors play a considerable role in mediating cellular injury of myelinated axons (see 1.1.5). However, little else is known about whether axon cylinders or glia/myelin may be vulnerable, nor have the source of endogenous glutamate or putative release mechanisms been identified. The studies in this thesis concentrate on these fundamental cellular mechanisms of white matter damage, a thorough understanding of which would be essential for efficient and logical design of neuroprotective intervention. The results of these basic studies may lead to the successful

development of therapeutic strategies, and guide future research aimed at developing effective clinical treatments.

1.4.1 Hypothesis:

During anoxic or traumatic CNS white matter injury, the rise of cytosolic Na^+ , together with depolarized membrane potential, causes reversal of the Na^+ -glutamate transporter, resulting in release of glutamate and subsequent injury to glia and myelin by overactivation of ionotropic glutamate receptors present in white matter tracts.

1.4.2 Objectives

The main goal of this project is to gain a greater understanding of the functional link between the movement of ions, such as influx of Na^+ and efflux of K^+ , and non-vesicular release of glutamate via reversal of Na^+ -dependent glutamate transporters. I also plan to explore the mechanisms and cellular targets of glutamate-mediated axonal injury in anoxia and trauma. The ultimate goal is to design a rational therapy aimed at the above-mentioned mechanisms, in the hope of blunting the deleterious effects of aberrant Na^+ and glutamate movement which cause irreversible tissue injury. There are three objectives in present study. The first two objectives involve normal uninjured myelinated axons (*Chapters 2 and 3*). I then move to the controlled *in vitro* injury models using 60 min anoxia and trauma using mechanical clip compression (*Chapter 4*).

#1 Excitotoxicity of spinal cord myelinated axons (*Chapter 2*)

Experiments were designed to investigate: 1) the susceptibility of spinal cord myelinated axons to exogenous excitotoxins such as glutamate, kainate or AMPA, 2) the locus of injury induced by excitotoxins i.e. axon cylinder, myelin sheath, glial cell bodies, and 3) cellular localization of glutamate receptors involved in the excitotoxicity.

#2 Induction of glutamate release by reverse transport (Chapter 3)

To study: 1) the altered ion gradients, such as increased $[\text{Na}^+]_i$ and $[\text{K}^+]_o$, induced release of endogenous (axonal, glial) glutamate by reverse Na^+ -dependent glutamate transport, 2) the deleterious effects of endogenous glutamate release by reverse transport, and protective effects of glutamate receptor antagonists and transport inhibitors, 3) cellular sources of endogenous glutamate released in white matter tracts.

#3 Glutamate release via reverse transport in injured axons (Chapter 4)

In this section, my studies are focused on: 1) the role of the excitatory amino acid in anoxic or traumatic axonal injury, 2) the mechanism of glutamate release in spinal cord axons injured by anoxia or mechanical trauma, and the identification of cell(s) responsible for glutamate release, 3) the distribution of glutamate transporter(s) in myelinated spinal cord white matter tracts.

Figure 1 General diagram showing multiple pathways involved in intracellular Ca^{2+} regulation. Ca^{2+} can enter a cell via voltage-gated Ca^{2+} channels, Ca^{2+} -permeable ionotropic receptors (such as AMPA/kainate glutamate receptor) and store-operated channels. The activation of metabotropic receptors (such as metabotropic glutamate receptor) leads to production of IP_3 and Ca^{2+} release from internal stores via IP_3 receptors. Ca^{2+} can also be released from internal Ca^{2+} stores via Ca^{2+} -induced Ca^{2+} release (ryanodine receptor) and mitochondrial Na^+ - Ca^{2+} exchanger. Cytoplasmic Ca^{2+} clearance occurs by Ca^{2+} extrusion via plasmalemmal Ca^{2+} pump and Na^+ - Ca^{2+} exchange as well as Ca^{2+} uptake into internal Ca^{2+} stores via SERCA and Ca^{2+} uniporter.

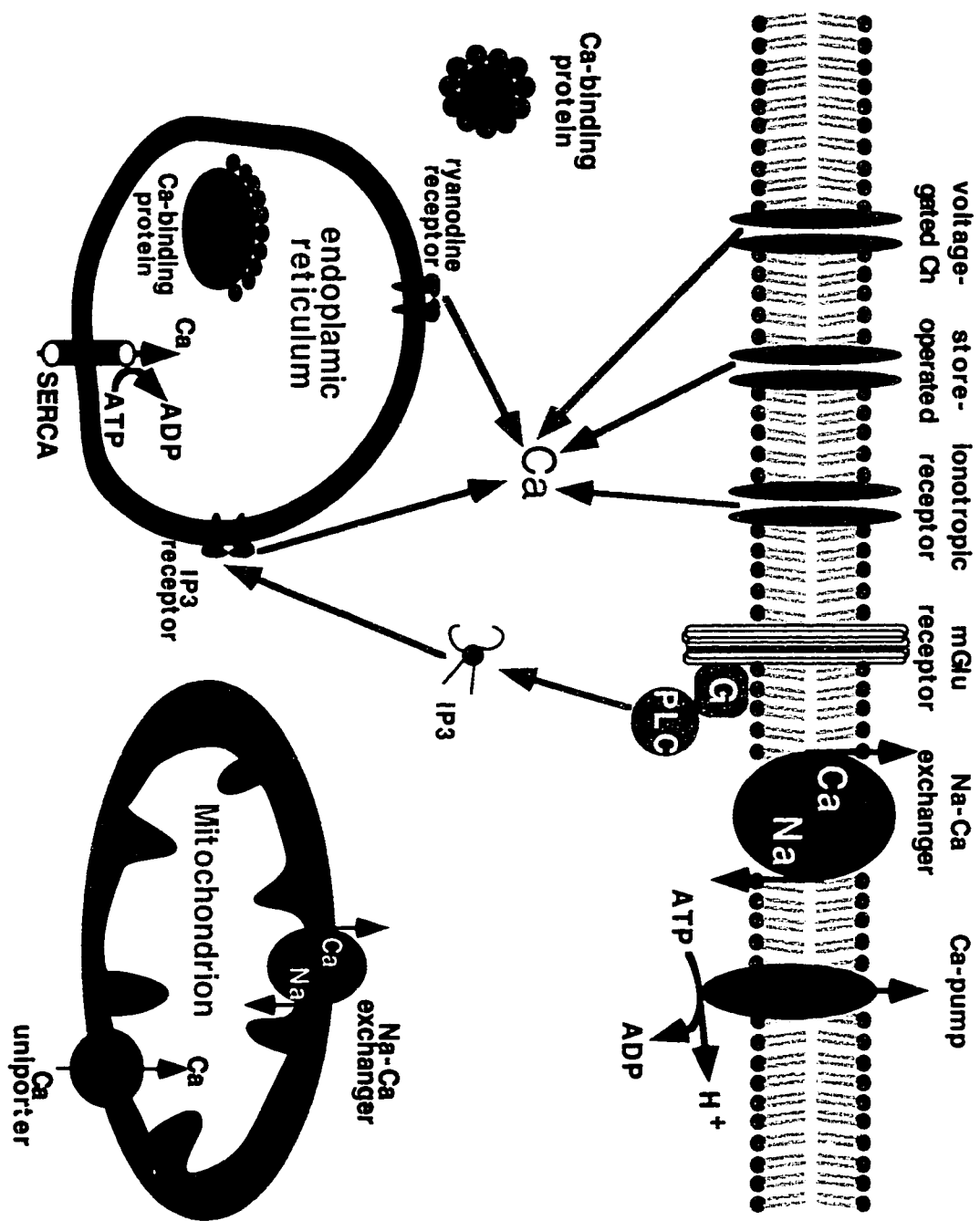


Figure 1

Figure 2 Structure of AMPA receptor subunits.

A: The transmembrane topology is shown along with the flip/flop alternatively spliced exon, and two ligand-binding domains (S1 and S2). Glycosylation sites are shown as trees in the N-terminal region.

B: The subunit has a large extracellular N-terminus domain, an intracellular C-terminus domain. AMPA receptors have only three transmembrane domains, M1, M3 and M4. The previously proposed transmembrane M2 does not span the membrane, but is only close to the intracellular surface within plasma membrane and makes a re-entrant loop (hairpin turn) within membrane. From the filled small square, we see two RNA editing sites, glutamine-(Q)to-arginine (R) at position 586 and arginine (R)-to-glycine(G) at position 743. The box around amino acids 744-781 shows the region where alternative splicing variants, flip and flop. Nine amino acids in the flip version are indicated by double arrows inside the box. When these amino acids are changed to those outside the box, the subunit becomes flop isoform.

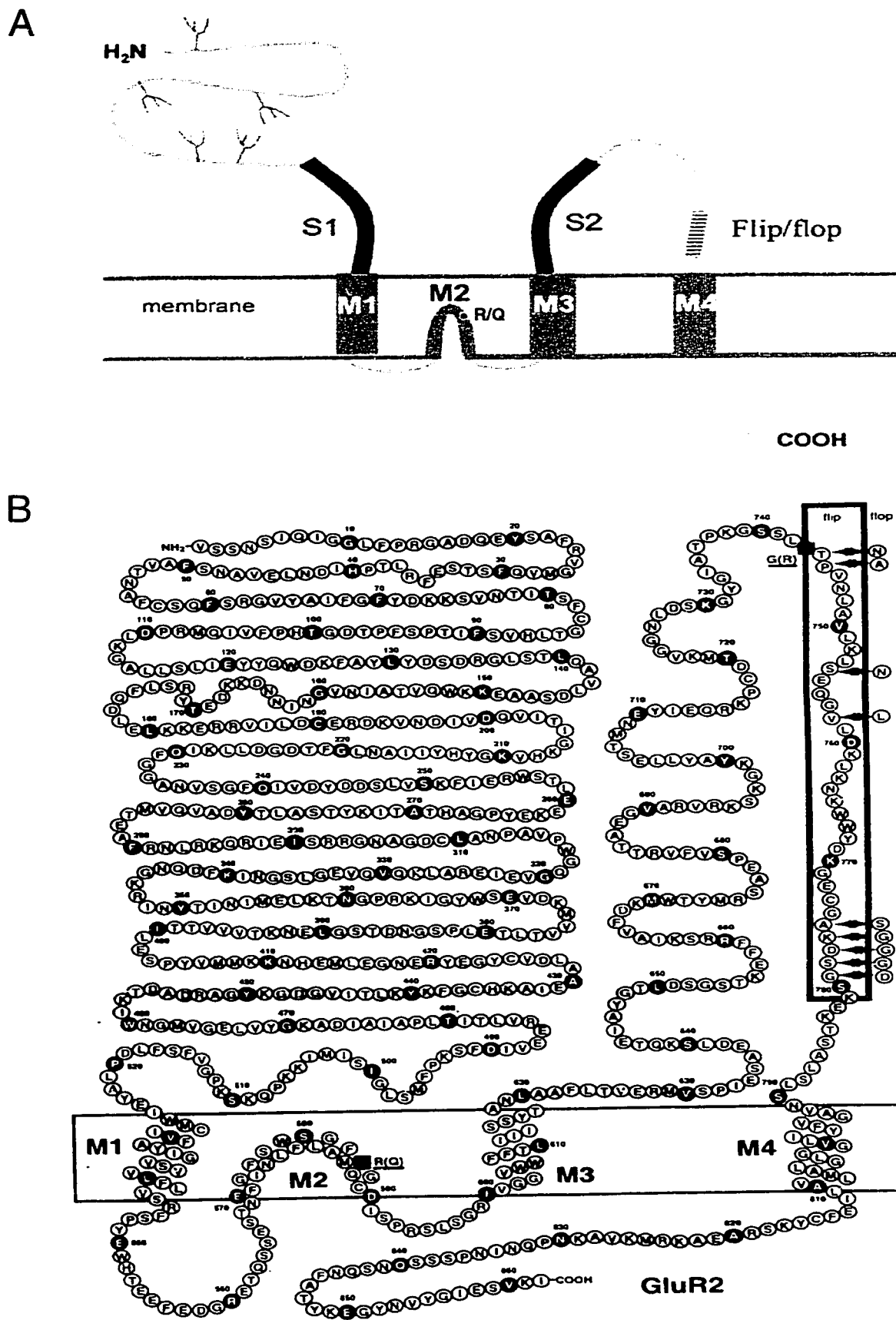


Figure 2

Chapter 2

Mechanisms of ionotropic glutamate receptor-mediated excitotoxicity in isolated spinal cord white matter

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ABSTRACT

Spinal cord injury involves a component of glutamate-mediated white matter damage, but the cellular targets, receptors and ions involved are poorly understood. Mechanisms of excitotoxicity were examined in an *in vitro* model of isolated spinal dorsal columns. Compound action potentials (CAPs) were irreversibly reduced to 43% of control after 3 h of 1 mM glutamate exposure at 37°C. AMPA (100 μ M) and kainate (500 μ M) had similar effects. Antagonists (1 mM kynurenic acid, 10 μ M NBQX, 30 μ M GYKI52466) were each equally protective against a glutamate challenge, improving mean CAP amplitude to $\approx$ 80% vs. $\approx$ 40% without antagonist. Joro spider toxin (0.75 μ M), a selective blocker of Ca^{2+} -permeable AMPA receptors, was also protective to a similar degree. Ca^{2+} -free perfusate virtually abolished glutamate-induced injury ($\approx$ 90% vs. $\approx$ 40%). MK-801 (10 μ M) had no effect. Glutamate caused damage (assayed immunohistochemically by spectrin breakdown products) to astrocytes and oligodendrocytes consistent with the presence of GluR2/3 and GluR4 in these cells. Myelin was also damaged by glutamate likely mediated by GluR4 receptors detected in this region. However axon cylinders were unaffected by glutamate, showing no increase in the level of spectrin breakdown. These data may guide the development of more effective treatment for acute spinal cord injury by addressing the additional excitotoxic component of spinal white matter damage.

Key words

Glutamate, excitotoxicity, AMPA receptor, spinal cord white matter, myelin, axon, glia, oligodendrocyte, astrocyte, spectrin, Joro spider toxin, GYKI52466, NBQX, MK-801, kainate.

INTRODUCTION

White matter tracts play the very important role of transmitting signals between neurons in the CNS. In the case of the spinal cord, disruption of axonal connections spanning even a small segment can result in severe and widespread disability involving functions distal to the lesion. Previous studies indicate that voltage-gated Na⁺ channels play an important role in anoxic and traumatic cellular injury of myelinated central axons (Agrawal and Fehlings, 1996; Imaizumi et al., 1997; Stutzmann et al., 1996; Stys, 1998; Stys et al., 1992; Teng and Wrathall, 1997). In addition, more recent reports indicate that CNS white matter injury is also dependent on excitotoxic mechanisms involving glutamate receptors of the AMPA/kainate class (Agrawal and Fehlings, 1997; Rosenberg et al., 1999; Wrathall et al., 1997). However, neither the mechanisms nor the cellular and subcellular targets of glutamate excitotoxicity in CNS white matter are well understood.

Oligodendrocytes and astrocytes have been shown to possess glutamate receptors of AMPA and kainate subtypes (Agrawal and Fehlings, 1997; Garcia-Barcina and Matute, 1996; Jensen and Chiu, 1993; Matute and Miledi, 1993; Matute et al., 1997; Steinhauser and Gallo, 1996). Persistent activation of these receptors causes injury to oligodendrocytes both in cell culture and *in vivo* (Matute, 1998; Matute et al., 1997; McDonald et al., 1998; Yoshioka et al., 1996; Yoshioka et al., 1995). Similarly, overactivation of AMPA receptors is very toxic and even lethal to astrocytes when receptor desensitization is blocked (David et al., 1996). In this study, using isolated rat spinal cord dorsal column slices, we examined the pharmacological features of excitotoxic injury in *in vitro* spinal cord white matter at physiological temperature, and

found that spinal cord white matter is markedly damaged by activation of AMPA receptors. We also explored the subcellular loci of injury, and observed that oligodendrocytes, astrocytes and particularly the myelin sheath, are targets for excitotoxic injury, with little evidence of damage to the axon cylinder *per se*. Our data extend previous observations that blockade of glutamate receptors is protective against white matter trauma or anoxia (Agrawal and Fehlings, 1997; Li et al., 1999; Wrathall et al., 1994) by elucidating which elements may be spared by, and which are unlikely to benefit from, glutamate receptor antagonists.

MATERIALS AND METHODS

Electrophysiology. Experimental procedures have been previously described (Li et al., 1999). Briefly, adult Long-Evans male rats (200-250 g) were deeply anesthetized with sodium pentobarbital, and a thoracic laminectomy performed. Rats were then perfused intra-aortically with 500 ml zero- Na^+ / zero- Ca^{2+} solution (in mM: choline chloride: 135, choline bicarbonate: 26, KCl: 1, KH_2PO_4 : 1.2, dextrose: 10 and EGTA: 1.0, bubbled with 95% O_2 / 5% CO_2). A 30 mm section of spinal cord was rapidly removed and placed in cold (4-6 °C) zero- Na^+ / zero- Ca^{2+} solution bubbled with 95% O_2 / 5% CO_2 . The spinal cord section was hemisected, the dorsal columns gently excised and placed in an interface recording chamber bathed in normal- Na^+ / zero- Ca^{2+} solution (mM: NaCl: 126, KCl: 3.0, MgSO_4 : 2.0, NaHCO_3 : 26, NaH_2PO_4 1.25, MgCl_2 : 2.0, dextrose: 10, EGTA: 0.5 mM) at room temperature bubbled with 95% O_2 / 5% CO_2 . The bath temperature was slowly raised to and maintained at 37 °C with a temperature controller (Model TC-102, Medical Systems Corp, Greenvale, NY), then the perfusate was switched to artificial cerebrospinal

fluid containing normal [Ca] ('aCSF', in mM: NaCl: 126, KCl: 3.0, MgSO₄: 2.0, NaHCO₃: 26, NaH₂PO₄ 1.25, CaCl₂: 2.0, dextrose: 10). Control recordings were taken 30 min after the temperature reached 37 °C in aCSF.

Propagated compound action potentials (CAPs) were evoked using a bipolar silver wire stimulating electrode placed on one end of the dorsal column and a constant voltage pulse (50 µs and typically 70 V) delivered once every 30 min. CAPs were recorded extracellularly at the opposite end using large-tipped glass microelectrodes filled with 150mM NaCl. To allow recording of multiple slices during a single experiment, the stimulation and recording sites were marked with a small amount of neutral red dye to allow accurate repositioning of the electrodes. Evoked CAPs were digitized, stored and analyzed using WaveTrak software without rectification (Stys, 1994). The functional integrity of the dorsal column was quantitated by measuring peak CAP amplitude.

Pharmacological agents. L-glutamic acid, MK801, NBQX (Sigma), kainic acid, kynurenic acid and Joro spider toxin (JSTX-3 tris(trifluoroacetate), RBI) were dissolved directly into aCSF. AMPA, cyclothiazide (Sigma) and N-methyl-D-aspartate (NMDA, Tocris) were first dissolved in NaOH (0.1 N for AMPA and cyclothiazide; 1 N for NMDA), and GYKI52466 (RBI) in 0.1N HCl, then added to aCSF to the desired final concentration. The pH of the solutions was maintained at 7.4. Glutamate receptor antagonists (MK801, NBQX, kynurenic acid, GYKI52466, JSTX-3) were applied beginning 30 min before addition of agonist (glutamate, kainate, AMPA).

Immunohistochemistry. We used quantitative confocal immunofluorescence to directly examine which dorsal column white matter elements are damaged by glutamate receptor activation. Rabbit antiserum raised against degenerated myelin basic protein ('anti-EP', a generous gift from Dr. Pat McGeer, University of British Columbia) was used to assay damage to the myelin sheath. This antibody stains myelin only in damaged, but not intact, white matter regions (Matsuo et al., 1997). Antiserum against spectrin breakdown products (a generous gift from Dr. Jon Durkin, National Research Council, Ottawa, Canada) was used to examine Ca^{2+} -dependent calpain-mediated degradation of the structural protein spectrin in axons (Hewitt et al., 1998; Isayama et al., 1991), and cell bodies and processes of oligodendrocytes and astrocytes. Spectrin is a ubiquitous cytoskeletal protein that is cleaved by calpain, itself activated by a rise in cytosolic $[\text{Ca}^{2+}]$. Thus calpain-cleaved spectrin breakdown is a reliable indicator of Ca^{2+} -dependent tissue injury in CNS ischemia and trauma (Roberts-Lewis et al, 1994; Buki et al, 1999). Following surgical preparation and spinal cord dissection as above, dorsal column slices were incubated in normal aCSF or 1 mM glutamate for 3 hours, and then fixed in 4% paraformaldehyde for 24 hours, and cryoprotected for 48 hrs in phosphate-buffered saline (PBS, pH7.4) containing 20% glycerol at 4 °C. Ends were sometimes gently teased to allow imaging of individual axons (e.g. Fig. 5C). Slices were then dissected into smaller pieces (approximately 3 x 2 x 0.5 mm), preincubated in 10% Triton X-100 for 30 min, followed by 4% normal goat serum (NGS) with 0.1% Triton X-100, and PBS for blocking for 1 hr at room temperature. After a single quick rinse in PBS, the sections were incubated for 24 hours at 4 °C with primary antiserum diluted in 2% NGS with

0.1% Triton X-100, PBS at a concentration of 1:100 for anti-EP, anti-spectrin breakdown, anti-mouse neurofilament 160 (Sigma) [marker for axon cylinders], anti-glial fibrillary acidic protein ('GFAP', Boehringer Mannheim) [astrocytes (Dusart et al., 1991)], and anti- 2',3'-cyclic-nucleotide 3'-phosphodiesterases (CNPase, Promega Corporation, Madison) a known cytoplasmic marker for oligodendrocytes and their putative progenitors (Braun et al., 1988; Trapp et al., 1988). Antibodies against GluR1, GluR2/3, GluR4 (Chemicon International Inc.) and GluR2 (Oncogene Research Products, Cambridge, MA) receptor subunits were used at 2-4 $\mu\text{g/ml}$. Following the primary antibody incubation, slices were rinsed three times in PBS for 30 minutes, then incubated for 1 hr with AlexaTM 594 goat anti-rabbit (1:200) and AlexaTM 488 goat anti-mouse (1:400, Molecular Probes) diluted in PBS with 2% NGS and 0.1% Triton X-100. Control sections were incubated with either the primary antisera omitted or secondary antibodies omitted. Images were collected on a Bio-Rad 1024 confocal laser scanning microscope with a 60X oil-immersion objective (Olympus). A minimum of 10 images collected from 2 to 3 sections were examined for each combination of markers, and representative images are shown. Digitized images were analyzed using NIH Image 1.61 (<http://rsb.info.nih.gov/nih-image/default.html>) on a Macintosh Power PC.

Statistics. All data are expressed as means $\pm$ standard deviation. Statistical differences were calculated by ANOVA with Dunnett's test for multiple comparisons with a common control group in the case of electrophysiological data. Student's t test was used for quantitative immunofluorescence data when only two groups were compared. Reported

n's represent number of individual dorsal column slices studied electrophysiologically, or the number of confocal image frames analyzed for fluorescence intensity.

RESULTS

Spinal cord white matter is vulnerable to excitotoxins

In control dorsal column slices perfused with normal aCSF, electrophysiological recording of CAPs showed less than 5% change in mean peak CAP amplitude during 3 hr of *in vitro* monitoring at physiological temperature (Fig. 1B) (Li et al., 1999). Slices incubated in 1 mM glutamate exhibited CAP amplitudes that were significantly decreased 90 min after the start of glutamate exposure in comparison with time-matched controls. At the end of a 3 hr glutamate application, mean CAP amplitude was reduced to $43 \pm 18\%$ of baseline CAP amplitude recorded at time zero, and was significantly reduced compared to time-matched controls ($p < 0.01$) (Fig.1A and B). Moreover, the glutamate-induced conduction failure did not recover after 1 hr wash with glutamate-free perfusate (Fig.2A and B). These results indicate that glutamate caused functional impairment of *in vitro* dorsal column white matter tracts at physiological temperature, which appears irreversible at least in the acute period. Similarly, the non-NMDA receptor agonists kainate (500 μ M) and AMPA (100 μ M), caused a significant attenuation of peak CAP amplitude to a degree similar to that induced by glutamate (Fig.2). This functional injury was also irreversible after 1 hr of wash. In contrast, a 3 hr exposure to 500 μ M NMDA (with 20 μ M glycine and in the absence of Mg^{2+} to maximize activation of NMDA receptors) had no effect (Fig.1A and B).

Excitotoxicity in dorsal columns is mediated via AMPA receptors and is Ca²⁺-dependent

Kynurenic acid (1mM), a broad spectrum blocker of both NMDA and AMPA/kainate receptors, applied 30min before glutamate exposure, significantly protected the dorsal column slices from glutamate toxicity (Fig.3A and B), supporting the notion that glutamate induced injury to spinal cord white matter is mediated via ionotropic glutamate receptors. Coapplication of MK-801 (10 μ M), a noncompetitive NMDA receptor antagonist, with glutamate, did not prevent glutamate-induced damage ($47 \pm 16\%$ vs $43 \pm 18\%$, Fig.3A and B), further supporting the notion that NMDA receptors play little if any role in glutamate toxicity. It is likely then that AMPA/kainate receptors mediate excitotoxic injury in spinal cord white matter. Enhancing AMPA receptor activation by reducing desensitization with cyclothiazide (100 μ M) (Mösbacher et al., 1994) in addition to glutamate, caused a more rapid decline in CAP amplitude over time, though the final degree of injury after 3 hrs of exposure was not significantly different from glutamate alone (Fig. 1C). The protective effect of the competitive AMPA/kainate receptor antagonist NBQX (10 μ M) (Sheardown et al., 1990) (CAP reduction to $79 \pm 23\%$ vs. $49 \pm 17\%$ without NBQX, $p < 0.01$, Fig. 3C), lends further support to the idea that glutamate-induced excitotoxicity in dorsal column white matter occurs primarily via overactivation of this subtype of ionotropic glutamate receptors. Fig. 4 illustrates the protective effect of GYKI52466 (30 μ M), a specific AMPA receptor antagonist (Paternain et al., 1995). This agent provided robust neuroprotection against AMPA/kainate receptor activation (CAP reduction to $82 \pm 10\%$ vs. $40 \pm 19\%$ without antagonist, $p < 0.01$), to a similar degree observed with the broader spectrum blockers

NBQX and kynurenic acid. Taken together, these findings indicate that AMPA, rather than NMDA or kainate receptors, are most responsible for glutamate-mediated dorsal column injury.

Certain AMPA receptors have been shown to display substantial permeability to Ca^{2+} (Dingledine et al., 1999; Ozawa et al., 1998) and influx of this divalent cation through these receptors contributes to neuronal death in several pathophysiological conditions, such as anoxia/ischemia and trauma (Pellegrini-Giampietro et al., 1997). Removal of Ca^{2+} from the perfusate or blocking Ca^{2+} influx with Joro spider toxin ($0.75 \mu\text{M}$), a blocker of Ca^{2+} -permeable AMPA receptors (Iino et al., 1996), protected dorsal columns from excitotoxicity (Figs. 3 and 4), to virtually the same degree as the AMPA antagonist GYKI52466 (mean CAP amplitude reduction to $89 \pm 16\%$ in Ca^{2+} -free, $83 \pm 4.6\%$ in JSTX-3 vs. $40 \pm 18\%$ in normal Ca^{2+} -containing aCSF, $p < 0.01$). These observations suggest that Ca^{2+} influx through Ca^{2+} -permeable AMPA receptors plays a major role in the genesis of excitotoxic damage in dorsal columns, probably by activation of Ca^{2+} -dependent degradative pathways such as calpains and phospholipases.

Glutamate-induced cellular damage is localized to myelin and glia

The previous results provide pharmacological evidence that glutamate, kainate and AMPA are toxic to spinal cord white matter, and this excitotoxicity is associated with Ca^{2+} influx principally through AMPA receptors. Physiological studies, while providing reliable functional measures, do not give detailed information about the subcellular loci of injury. We used immunocytochemistry with specific antiserum raised against

degenerated myelin basic protein (Matsuo et al., 1997) and calpain-cleaved breakdown products of the structural protein spectrin (Hewitt et al., 1998), to examine which white matter elements are vulnerable to excitotoxins. Fig.5 shows representative confocal images of dorsal column white matter incubated with normal aCSF (left panels) or 1mM glutamate for 3 hrs (right panels). Double staining with neurofilament (green) to outline axon cylinders, and degenerated myelin basic protein (red), showed that a 3 hr glutamate exposure induced significant damage to the myelin sheath (red signal surrounding axon cylinders, arrowhead, panels B and C). In contrast, time-matched control sections without glutamate displayed virtually no myelin damage (panel A).

Immunostaining for spectrin breakdown products (SBP) allows detection of structural damage to the cytoskeleton in axon cylinders and glia. Astrocytes and oligodendrocytes were distinguished using GFAP (Dusart et al., 1991) and CNPase (Trapp et al., 1998), respectively. Double staining for cellular marker proteins (i.e. CNPase, GFAP and neurofilament) and SBP revealed that control sections displayed low levels of SBP in the cell bodies and processes of oligodendrocytes, axon cylinders and astrocytes, best seen in the separated gray scale images in Fig. 6 C, G and K. These degenerated spectrin products may represent normal basal turnover of structural proteins, induction of mild injury by the procedure of tissue isolation and *in vitro* incubation, and/or mild non-specific staining by the antiserum. In contrast, a 3 hr exposure to 1 mM glutamate induced significant damage to oligodendroglial and astrocytic cell bodies and processes, as shown by the red staining for SBP or yellow signal colocalizing SBP and CNPase/GFAP (Fig. 5 E and G). Notably, there was no detectable rise of SBP in

axoplasm after glutamate treatment (Fig. 5 I and 6 H). Fig. 6 illustrates separated gray scale images of sections double stained for standard markers and SBP in control and glutamate-treated groups. The increase in SBP fluorescence in oligodendrocytes and astrocytes in the glutamate-treated slices is shown quantitatively in the accompanying bar graphs, with significant increases observed in cytosolic regions of oligodendrocytes (48 ± 9 in glutamate group vs. 38 ± 7 in controls, $P < 0.01$, Fig. 6 left bar graph) and astrocytes (58 ± 11 vs. 31 ± 2 in controls, $p < 0.001$, Fig. 6 right bar graph). In contrast, glutamate did not induce any detectable structural injury, as estimated by spectrin breakdown, in axon cylinders even after 3 hr of exposure; SBP fluorescence was identical in treated vs. control sections (36 ± 2 vs. 36 ± 1 , respectively, $p = 0.879$, Fig. 6 middle bar graph).

AMPA receptors are expressed in oligodendrocytes, astrocytes and myelin of spinal cord white matter

Our data suggest that overactivation of Ca^{2+} - permeable AMPA receptors by excitotoxins in dorsal column white matter results in significant functional and structural damage to various cellular components including oligodendrocytes, astrocytes and the myelin sheath. Fig. 7 shows representative immunohistochemical sections stained for different AMPA receptor subunits. GluR4 was the most ubiquitous subunit, present in oligodendrocytes (panels G and H) and astrocytes (panel F), with astrocytic processes associated with capillaries being particularly rich in this receptor subunit. Axoplasm also contained GluR4 (panel E), and interestingly, the myelin sheath displayed noticeable GluR4 label as well (panel H). The weaker GluR4 signal in the axon cylinder in panel H is obscured by the much stronger neurofilament label, with a strong green MBP signal

obscuring GluR4 in the myelin in panel E; separated images (not shown), and particularly counterstains that are not overlapping (such as MBP in E and neurofilament in H) clearly indicate the presence of this subunit in both the axoplasm and myelin. The latter was devoid of isoforms other than GluR4. In contrast, with the exception of astrocytes, GluR1 was largely absent to any significant degree in dorsal column white matter elements. The combination of GluR2 and GluR2/3 antisera allowed us to distinguish between these two subunits. Given that GluR2 was not seen in glia, myelin or axon cylinders (results not shown), GluR2/3 positivity in oligodendrocytes and astrocytes (panels C and D) indicates the presence of GluR3 in these cells. To ensure that the total absence of GluR2 label was not artifactual, positive controls were done in spinal gray matter, known to contain GluR2-positive neurons (Grossman et al., 1999), where unequivocal neuronal staining was observed (not shown). GluR1 was weakly present in astrocytes and possibly in axon cylinders as well (faint signal outside of GFAP-positive regions in panel B; supported by neurofilament double staining [not shown]). Table 1 contains a summary of AMPA receptor subunit distributions in dorsal columns observed in our experiments.

DISCUSSION

White matter tracts of the mammalian spinal cord serve the critical function of conducting signal traffic to and from the brain. Traumatic and ischemic damage to the cord often results in major clinical disability, most of which is due to dysfunction of white matter tracts rather than gray matter regions (Blight and Decrescito, 1986; Noble and Wrathall, 1989). Recent reports suggest that, perhaps surprisingly, glutamate-

dependent mechanisms appear to play a role in this tissue that is devoid of synaptic elements. However, neither the source of glutamate, release mechanism nor cellular targets are known. In this study, using an isolated *in vitro* spinal white matter tract, we studied the glutamate receptors involved, the cellular targets of excitotoxicity and the ionic dependence. We observed that overactivation of mainly the AMPA receptor subclass by glutamate or related agonists, caused significant irreversible functional injury. Excitatory amino acids were directly toxic to dorsal column slices, and this damage was not dependent on adjacent gray matter or vascular supply, which were absent in our model.

Electrophysiological recordings showed that CAP amplitude was irreversibly reduced to less than half of control following 2 to 3 hours of agonist exposure at physiological temperature, with conduction impairment appearing as early as 30 min when AMPA receptor desensitization was blocked (Fig. 1). In an earlier study, Agrawal and Fehlings (1997) reported that both AMPA and kainate reduced the amplitude of CAPs recorded from *in vitro* spinal white matter, but the degree of conduction impairment was much more modest compared to our results and was reversible, in contrast to our findings where no recovery was detectable after wash of the excitotoxin (Fig. 2). We believe that the elevated temperature at which we conducted our experiments induced significantly more excitotoxic injury compared to the hypothermic conditions used previously, indicating that glutamate-dependent injury may be far more important in white matter than previously appreciated.

Using selective agonists and antagonists, we concluded that the AMPA, and not the NMDA, receptor class is largely responsible for glutamate-mediated injury to spinal cord white matter. This is consistent with previous observations of neuroprotection afforded by AMPA receptor antagonists in models of *in vitro* and *in vivo* spinal cord injury and anoxia (Agrawal and Fehlings, 1997; Li et al., 1999; Wrathall et al., 1994), but is in contrast to neurons, where both classes of receptors are known to cause damage (Choi, 1994; Choi and Hartley, 1993). Our data also indicate that the AMPA receptor-mediated injury is highly dependent on influx of extracellular Ca^{2+} : removal of this ion from the perfusate allowed dorsal column slices to withstand a 3 hr kainate challenge with virtually no noticeable reduction in excitability (Fig. 4). Together with the highly protective effect of Joro spider toxin, a selective blocker of Ca^{2+} -permeable AMPA receptors (Iino et al., 1996), our data strongly suggest that Ca^{2+} influx directly through AMPA receptors plays an important role. However, alternate Ca^{2+} entry routes cannot be ruled out. For instance, AMPA receptor-mediated cellular Na^+ loading and depolarization may secondarily induce Ca^{2+} entry through voltage-gated Ca^{2+} channels or reverse Na^+ - Ca^{2+} exchange (Hack and Balazs, 1995; Liu et al., 1997).

Electrophysiological measurements yield quantitative information about the overall functional state of tissue, but do not provide insight into which white matter elements are vulnerable to glutamate-mediated toxicity. We used confocal microscopy and immunohistochemistry with antisera raised against damaged, but not intact, structural proteins including myelin basic protein and spectrin. As shown in Figs. 5 and 6, glutamate caused significant structural injury to glial elements, with no detectable effect

on axon cylinders. Thus only myelin, astrocytes and oligodendrocytes appeared to be vulnerable to excitotoxins. Our findings are consistent with recent reports showing that both oligodendrocytes and astrocytes are damaged by overactivation of AMPA/kainate receptors. AMPA or kainate causes rapid cell death in cultured oligodendrocytes in a Ca^{2+} -dependent manner (Matute et al., 1997; McDonald et al., 1998). Similarly, application of AMPA or kainate to white matter *in vivo* causes widespread death of oligodendrocytes (Matute, 1998; Matute et al., 1997). Cultured astrocytes are relatively resistant to excitatory amino acid toxins (Choi et al., 1987; Koh et al., 1990). However, Ca^{2+} permeable AMPA and kainate subunits of glutamate receptors have been identified in these cells (Agrawal and Fehlings, 1997; Burnashev et al., 1992; Garcia-Barcina and Matute, 1996; Seifert and Steinhauser, 1995), and damage can be induced when receptor desensitization is pharmacologically blocked (David et al., 1996). Our results demonstrate that damage to astrocytes can be detected following a 3 hr exposure to glutamate even without inhibition of receptor desensitization (Figs. 5 and 6). Previous reports indicate incompletely desensitizing responses in cultured astrocytes to glutamate application (Blankenfeld et al., 1995). It is therefore likely that the residual permeability induced by glutamate in our tissue was sufficient to induce injury after a 3 hr exposure. While we believe that AMPA receptors play a prominent role, a component of kainate receptor activation in astrocytic and/or oligodendroglial injury cannot be excluded since we did not examine the effects of agonists selective for this receptor subtype.

An interesting observation from our studies was glutamate-induced structural damage to the myelin sheath itself (Fig. 5B & C). Myelin plays a critical role in sustaining saltatory

conduction, with damage to the sheath resulting in slowing or complete failure of conduction (Waxman, 1992). In this study, excitotoxins caused impairment of conduction beginning as early as 30 min after drug application, in the absence of any detectable injury to the axon cylinder *per se*. This raises the strong possibility that functional white matter impairment secondary to excitotoxic exposure is largely, if not exclusively, due to damage to glial elements, particularly the myelin sheath given the rapidity of the effect on conduction. While we cannot exclude the possibility that myelin damage was secondary to injury of the parent oligodendrocyte, the rapid disturbance of dorsal column excitability by glutamate suggests that excitotoxins exerted a direct effect on the myelin sheath. This hypothesis is further supported by the finding that GluR4, but not GluR2, receptor subunits are present in myelin, indicating that the sheath itself may respond to ambient glutamate, and if excessively stimulated, may suffer a toxic Ca^{2+} influx directly through Ca^{2+} -permeable AMPA receptors.

As summarized in Table 1, we also found GluR3 and GluR4 subunits in astrocytes, oligodendrocytes and axoplasm. Our findings in glia are consistent with observations from other groups, where PCR studies and immunocytochemistry have directly demonstrated the expression of AMPA/kainate receptors in white matter glia (Agrawal and Fehlings, 1997; Garcia-Barcina and Matute, 1996; Jensen and Chiu, 1993; Matute, 1998; Matute and Miledi, 1993; Matute et al., 1997; McDonald et al., 1998; Patneau et al., 1994; Steinhauser and Gallo, 1996). For example, oligodendrocytes from optic nerve express GluR3 and GluR4 subunits of the AMPA receptor and GluR6-7 and KA1-2 subunits of the kainate receptor, but not GluR2 (Matute et al., 1997), which is congruent

with our findings of positive immunoreactivity for GluR4 and GluR2/3, but not GluR2, in this cell. The faint GluR1 immunoreactivity seen in dorsal column astrocytes is consistent with localization exclusively to this cell type in bovine corpus callosum (Garcia-Barcina and Matute, 1998). The significance of GluR2/3 and 4 immunoreactivity in axoplasm is unclear, and it is possible that subunits are being transported for insertion at the terminals. We cannot however exclude that some receptors are by inference present in the axolemma. If so, unlike glia and myelin, the density must be low enough so that activation of these receptors did not cause any detectable damage to the axon cylinder.

In summary we have shown that isolated spinal dorsal columns are vulnerable to irreversible excitotoxic injury that is dependent on AMPA receptor activation and Ca^{2+} influx from the extracellular space. The physiological role of glutamate receptors in white matter is not known, but may involve activity-dependent signaling between axons and surrounding glia (Chiu and Kriegler, 1994). Our finding of AMPA receptor subunits directly on myelin raises the intriguing possibility that axonal activity might directly modulate the metabolism and structure of the sheath itself, independently of or in addition to effects from the parent soma. During anoxia/ischemia or trauma, this mechanism, overdriven by ionic deregulation, may lead to irreversible injury. We have recently shown that glutamate is released from axon cylinders via reverse Na^+ -dependent glutamate transport during *in vitro* anoxia and trauma causing disruption of the myelin sheath in an AMPA receptor-dependent manner (Li et al., 1999). It is very likely that studies demonstrating neuroprotective effects of AMPA/kainate antagonists in models of

spinal cord injury conferred functional protection by sparing glia and myelin (Agrawal and Fehlings, 1997; Wrathall et al., 1994; Wrathall et al., 1997); this is supported by a recent morphological study showing sparing of glial elements by the AMPA/kainate antagonist NBQX after spinal cord injury (Rosenberg et al., 1999). An additional important injury mechanism in myelinated axons also involves axoplasmic Ca^{2+} overload mediated by reverse Na^{+} - Ca^{2+} exchange (Imaizumi et al., 1997; Stys, 1998; Stys and LoPachin, 1998). Taken together, it is possible that injury to central myelinated axons proceeds along two parallel routes, with Ca^{2+} influx through reverse Na^{+} - Ca^{2+} exchange causing damage to the axon cylinder, whereas glia and myelin suffer Ca^{2+} -dependent damage that is instead mediated by an excitotoxic mechanism.

REFERENCES

Agrawal SK, Fehlings MG (1996) Mechanisms of secondary injury to spinal cord axons in vitro - role of Na^+ , Na^+ - K^+ -ATPase, the Na^+ - H^+ exchanger, and the Na^+ - Ca^{2+} exchanger. *J Neurosci* 16:545-552.

Agrawal SK, Fehlings MG (1997) Role of NMDA and non-NMDA ionotropic glutamate receptors in traumatic spinal cord axonal injury. *J Neurosci* 17:1055-1063.

Blankenfeld GV, Enkvist K, Kettenman H (1995) Gamma-aminobutyric acid and glutamate receptors. In: Neuroglia (H. Kettenman and B. R. Ransom eds), pp 335-345. New York: Oxford.

Blight AR, Decrescito V (1986) Morphometric analysis of experimental spinal cord injury in the cat: the relation of injury intensity to survival of myelinated axons. *Neuroscience* 19:321-41.

Braun PE, Sandillon F, Edwards A, Matthieu JM, Privat A (1988) Immunocytochemical localization by electron microscopy of 2'3'-cyclic nucleotide 3'-phosphodiesterase in developing oligodendrocytes of normal and mutant brain. *J Neurosci* 8:3057-3066

Buki A, Siman R, Trojanowski JQ, Povlishock JT (1999) The role of calpain-mediated spectrin proteolysis in traumatically induced axonal injury. *J Neuropathol Exp Neurol* 58:365-375.

Burnashev N, Khodorova A, Jonas P, Helm PJ, Wisden W, Monyer H, Seeburg PH, Sakmann B (1992) Calcium-permeable AMPA-kainate receptors in fusiform cerebellar glial cells. *Science* 256:1566-70.

Chiu SY, Kriegler S (1994) Neurotransmitter-mediated signaling between axons and glial cells. *Glia* 11:191-200.

Choi DW (1994) Glutamate receptors and the induction of excitotoxic neuronal death. *Prog Brain Res* 100:47-51.

Choi DW, Hartley DM (1993) Calcium and glutamate-induced cortical neuronal death. *Res Publ Assoc Res Nerv Ment Dis* 71:23-34.

Choi DW, Maulucci GM, Kriegstein AR (1987) Glutamate neurotoxicity in cortical cell culture. *J Neurosci* 7:357-68.

David JC, Yamada KA, Bagwe MR, Goldberg MP (1996) AMPA receptor activation is rapidly toxic to cortical astrocytes when desensitization is blocked. *J Neurosci* 16:200-9.

Dingledine R, Borges K, Bowie D, Traynelis SF (1999) The glutamate receptor ion channels. *Pharmacol Rev* 51:7-61.

Dusart I, Marty S, Peschanski M (1991) Glial changes following an excitotoxic lesion in the CNS--II. Astrocytes. *Neuroscience* 45:541-9.

Garcia-Barcina JM, Matute C (1996) Expression of kainate-selective glutamate receptor subunits in glial cells of the adult bovine white matter. *Eur J Neurosci*. 8:2379-2387.

Garcia-Barcina JM, Matute C (1998) AMPA-selective glutamate receptor subunits in glial cells of the adult bovine white matter. *Brain Res Mol Brain Res* 53:270-6.

Grossman SD, Wolfe BB, Yasuda RP, Wrathall JR (1999) Alterations in AMPA receptor subunit expression after experimental spinal cord contusion injury. *J Neurosci* 19:5711-5720.

Hack N, Balazs R (1995) Properties of AMPA receptors expressed in rat cerebellar granule cell cultures: Ca^{2+} influx studies. *J Neurochem* 65:1077-84.

Hewitt KE, Lesiuk HJ, Tauskela JS, Morley P, Durkin JP (1998) Selective coupling of μ -calpain activation with the NMDA receptor is independent of translocation and autolysis in primary cortical neurons. *J Neurosci Res* 54:223-232.

Iino M, Koike M, Isa T, Ozawa S (1996) Voltage-dependent blockage of Ca^{2+} -permeable AMPA receptors by joro spider toxin in cultured rat hippocampal neurones. *J Physiol (Lond)* 496:431-7.

Imaizumi T, Kocsis JD, Waxman SG (1997) Anoxic injury in the rat spinal cord - pharmacological evidence for multiple steps in Ca^{2+} -dependent injury of the dorsal columns. *J Neurotrauma* 14:299-311.

Isayama T, Goodman SR, Zagon IS (1991) Spectrin isoforms in the mammalian retina. *J Neurosci* 11:3531-3538.

Jensen AM, Chiu SY (1993) Expression of glutamate receptor genes in white matter: developing and adult rat optic nerve. *J Neurosci* 13:1664-75.

Koh JY, Goldberg MP, Hartley DM, Choi DW (1990) Non-NMDA receptor-mediated neurotoxicity in cortical culture. *J Neurosci* 10:693-705.

Li S, Mealing GA, Morley P, Stys PK (1999) Novel injury mechanism in anoxia and trauma of spinal cord white matter: glutamate release via reverse Na^{+} -dependent glutamate transport. *J Neurosci* 19:RC16(1-9).

Liu HN, Molina-Holgado E, Almazan G (1997) Glutamate-stimulated production of inositol phosphates is mediated by Ca^{2+} influx in oligodendrocyte progenitors. *Eur J Pharmacol* 338:277-87.

Matsuo A, Lee GC, Terai K, Takami K, Hickey WF, McGeer EG, McGeer PL (1997) Unmasking of an unusual myelin basic protein epitope during the process of myelin degeneration in humans: a potential mechanism for the generation of autoantigens. *Am J Pathol* 150:1253-1266.

Matute C (1998) Characteristics of acute and chronic kainate excitotoxic damage to the optic nerve. *Proc Natl Acad Sci U S A* 95:10229-34.

Matute C, Miledi R (1993) Neurotransmitter receptors and voltage-dependent Ca^{2+} channels encoded by mRNA from the adult corpus callosum. *Proc Natl Acad Sci U S A* 90:3270-4.

Matute C, Sanchez-Gomez MV, Martinez-Millan L, Miledi R (1997) Glutamate receptor-mediated toxicity in optic nerve oligodendrocytes. *Proc Natl Acad Sci U S A* 94:8830-8835.

McDonald JW, Althomsons SP, Hyrc KL, Choi DW, Goldberg MP (1998) Oligodendrocytes from forebrain are highly vulnerable to AMPA/kainate receptor-mediated excitotoxicity. *Nat Med* 4:291-297.

Mosbacher J, Schoepfer R, Monyer H, Burnashev N, Seeburg PH, Ruppersberg JP (1994) A molecular determinant for submillisecond desensitization in glutamate receptors. *Science* 266:1059-62.

Noble LJ, Wrathall JR (1989) Correlative analyses of lesion development and functional status after graded spinal cord contusive injuries in the rat. *Exp Neurol* 103:34-40.

Ozawa S, Kamiya H, Tsuzuki K (1998) Glutamate receptors in the mammalian central nervous system. *Prog Neurobiol* 54:581-618.

Paternain AV, Morales M, Lerma J (1995) Selective antagonism of AMPA receptors unmasks kainate receptor-mediated responses in hippocampal neurons. *Neuron* 14:185-189.

Patneau DK, Wright PW, Winters C, Mayer ML, Gallo V (1994) Glial cells of the oligodendrocyte lineage express both kainate- and AMPA-preferring subtypes of glutamate receptor. *Neuron* 12:357-71.

Pellegrini-Giampietro DE, Gorter JA, Bennett MV, Zukin RS (1997) The GluR2 (GluR-B) hypothesis: Ca^{2+} -permeable AMPA receptors in neurological disorders. *Trends Neurosci* 20:464-70.

Roberts-Lewis JM, Savage MJ, Marcy VR, Pinsker LR, Siman R (1994) Immunolocalization of calpain I-mediated spectrin degradation to vulnerable neurons in the ischemic gerbil brain. *J Neurosci* 14:3934-3944.

Rosenberg LJ, Teng YD, Wrathall JR (1999) 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f)quinoxaline reduces glial loss and acute white matter pathology after experimental spinal cord contusion. *J Neurosci* 19:464-475.

Seifert G, Steinhauser C (1995) Glial cells in the mouse hippocampus express AMPA receptors with an intermediate Ca^{2+} permeability. *Eur J Neurosci* 7:1872-81.

Sheardown MJ, Neilsen EO, Hansen AJ, Jacobsen P, Honoré T (1990) 2,3-Dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline: a neuroprotectant for cerebral ischemia. *Science* 247:571-574.

Steinhauser C, Gallo V (1996) News on glutamate receptors in glial cells. *Trends Neurosci* 19:339-45.

Stutzmann JM, Pratt J, Boraud T, Gross C (1996) The effect of riluzole on post-traumatic spinal cord injury in the rat. *Neuroreport* 7:387-92.

Stys PK (1994) WaveTrak: a data acquisition system and waveform database for the Macintosh. *Sci. Comput. Automation* 10:19-24.

Stys PK (1998) Anoxic and ischemic injury of myelinated axons in CNS white matter: from mechanistic concepts to therapeutics. *J Cereb Blood Flow Metab* 18:2-25.

Stys PK, LoPachin RM (1998) Mechanisms of ion flux in anoxic myelinated CNS axons. *Neuroscience* 82:21-32.

Stys PK, Waxman SG, Ransom BR (1992) Ionic mechanisms of anoxic injury in mammalian CNS white matter: role of Na⁺ channels and Na⁺-Ca²⁺ exchanger. *J Neurosci* 12:430-439.

Teng YD, Wrathall JR (1997) Local blockade of sodium channels by tetrodotoxin ameliorates tissue loss and long-term functional deficits resulting from experimental spinal cord injury. *J Neurosci* 17:4359-4366.

Trapp BD, Bernier L, Andrews SB, Colman DR (1988) Cellular and subcellular distribution of 2',3'-cyclic nucleotide 3'-phosphodiesterase and its mRNA in the rat central nervous system. *J Neurochem* 51:859-868.

Trapp BD, Peterson J, Ransohoff RM, Rudick R, Mork S, Bo L (1998) Axonal transection in the lesions of multiple sclerosis. *N Engl J Med* 338:278-285.

Waxman SG (1992) Demyelination in spinal cord injury and multiple sclerosis: what can we do to enhance functional recovery? J Neurotrauma 9 (Suppl 1): S105-17.

Wrathall JR, Choiniere D, Teng YD (1994) Dose-dependent reduction of tissue loss and functional impairment after spinal cord trauma with the AMPA/kainate antagonist NBQX. J Neurosci 14:6598-6607.

Wrathall JR, Teng YD, Marriott R (1997) Delayed antagonism of AMPA/kainate receptors reduces long-term functional deficits resulting from spinal cord trauma. Exp Neurol 145:565-73.

Yoshioka A, Bacskai B, Pleasure D (1996) Pathophysiology of oligodendroglial excitotoxicity. J Neurosci Res 46:427-37.

Yoshioka A, Hardy M, Younkin DP, Grinspan JB, Stern JL, Pleasure D (1995) Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors mediate excitotoxicity in the oligodendroglial lineage. J Neurochem 64:2442-8.

Table 1 Distribution of AMPA receptor subunits in rat dorsal columns

	GluR1	GluR2	GluR2/3	GluR4
Axoplasm	-/+	-	++	+++
Myelin	-	-	-	++
Oligo	-	-	++	++
Astrocyte	+	-	++	+++*

Absent: -
Weak: +
Moderate: ++
Strong: +++

*especially pronounced around capillaries

Figure 1 Effect of glutamate (Glut), kainate (KA) or NMDA on excitability of *in vitro* dorsal column slices.

(A) Representative CAP tracings after 180 min of exposure to agonist.

(B) Bar graph showing quantitatively changes in mean peak CAP amplitudes (normalized to 100% at time zero) under various treatment conditions. Controls remained stable for 180 min at 37 °C. Exposure to glutamate (1 mM) or kainate (500 µM) significantly reduced CAP amplitude to $\approx$ 40% of control after 180 min. In contrast, NMDA (500 µM) had no effect. * $P < 0.05$, ** $P < 0.01$ compared to time-matched controls.

(C) Addition of cyclothiazide (100 µM), an inhibitor of AMPA receptor desensitization, caused a more rapid decay of mean CAP amplitude, although the final degree of injury was not different at the end of 180 min. * $P < 0.05$, ** $P < 0.01$ compared to time-matched readings in glutamate alone.

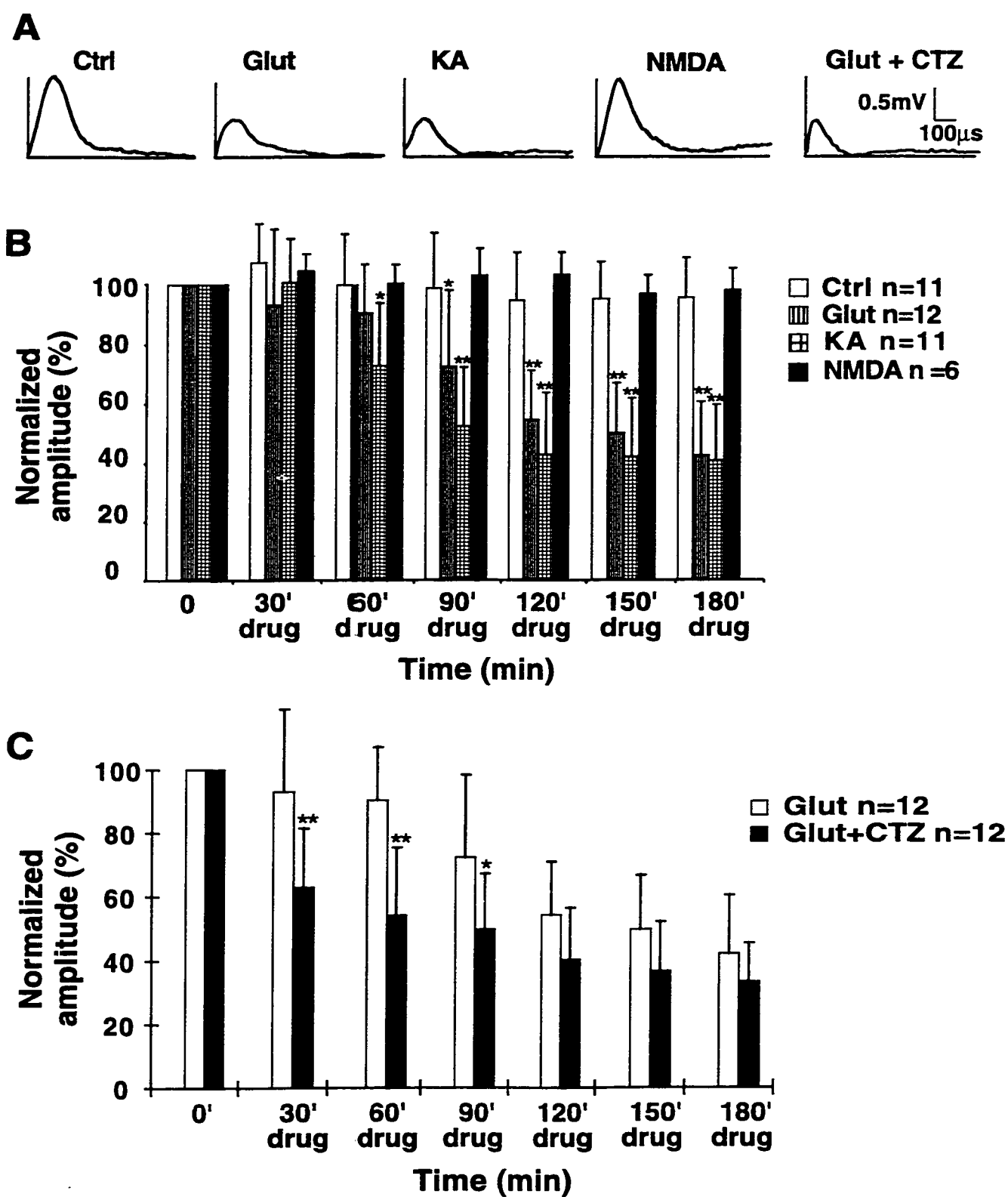


Figure 1

Figure 2 Effect of glutamate (Glut), kainate (KA) or AMPA followed by wash on excitability of *in vitro* dorsal column slices.

(A) Representative CAP tracings after a 120 min exposure to agonist followed by a 60 min wash.

(B) Bar graph showing reduction of normalized mean peak CAP amplitude during exposure to glutamate (1 mM), kainate (500 μ M) or AMPA (100 μ M). Impaired conduction was evident as early as 30 min. All three agents reduced excitability to the same degree after a 120 min exposure. No evidence of recovery was observed following 60 min of wash indicating irreversible excitotoxic injury to the tissue. * $P < 0.05$, ** $P < 0.01$ compared to time-matched controls.

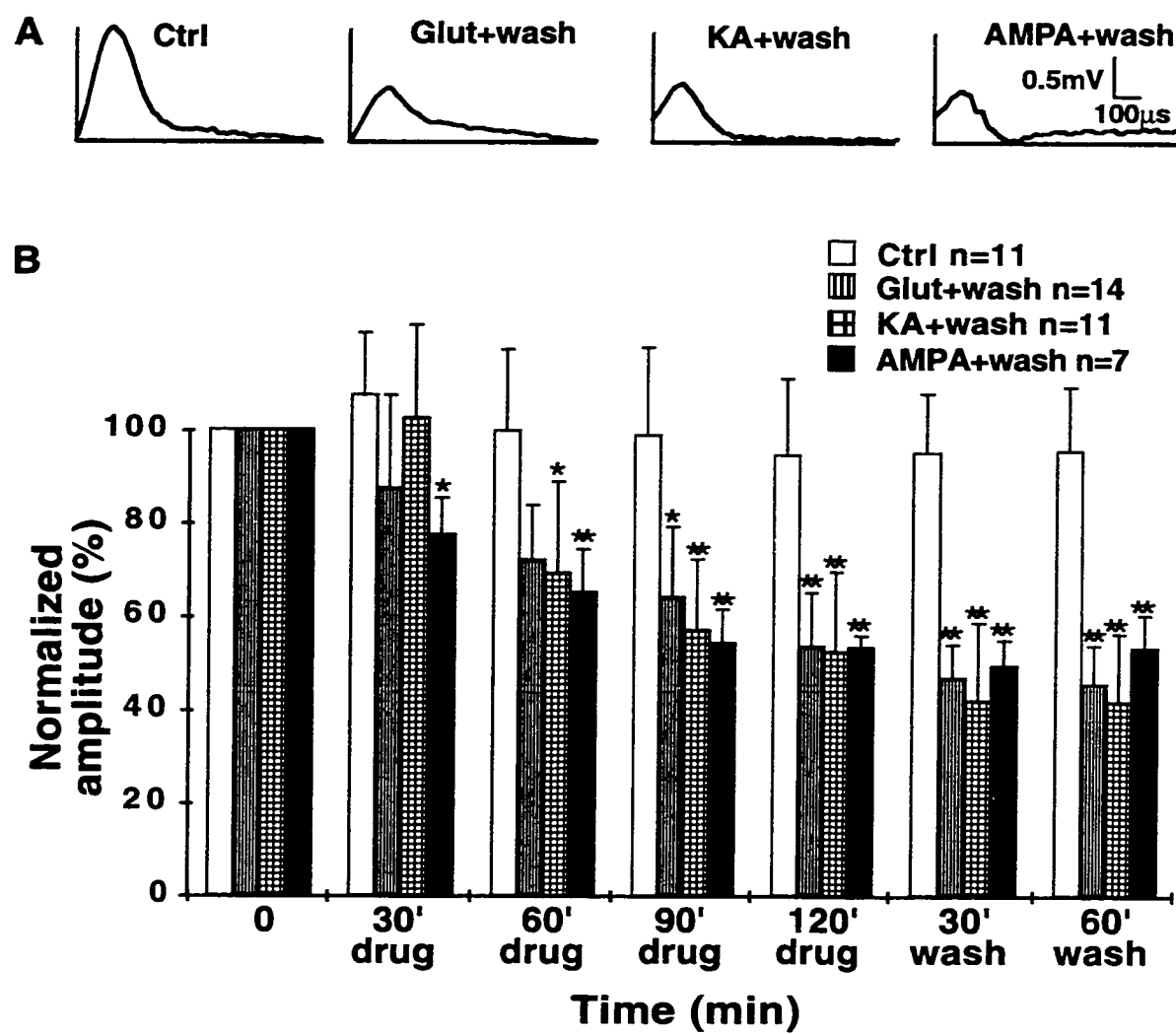


Figure 2

Figure 3 Effects of ionotropic glutamate receptor antagonists on glutamate toxicity in dorsal columns.

(A) Representative CAP tracings after a 180 min exposure to glutamate or glutamate + antagonist.

(B) Glutamate (1 mM) alone causes significant functional injury to isolated dorsal columns as shown by the reduction of normalized mean CAP amplitudes (white bars). The broad spectrum ionotropic glutamate receptor antagonist kynurenic acid ('Kyn', 1 mM) or Joro spider toxin ('JSTX', 0.75 μ M), a selective inhibitor of Ca²⁺-permeable AMPA receptors, each significantly protected the tissue from glutamate toxicity. Blocking NMDA receptors with MK-801 (10 μ M) was not protective.

(C) The AMPA/kainate receptor blocker NBQX (10 μ M) was also protective against glutamate and the desensitization inhibitor cyclothiazide ('CTZ', 100 μ M).

*P<0.05, **P<0.01 compared to time-matched readings without antagonist.

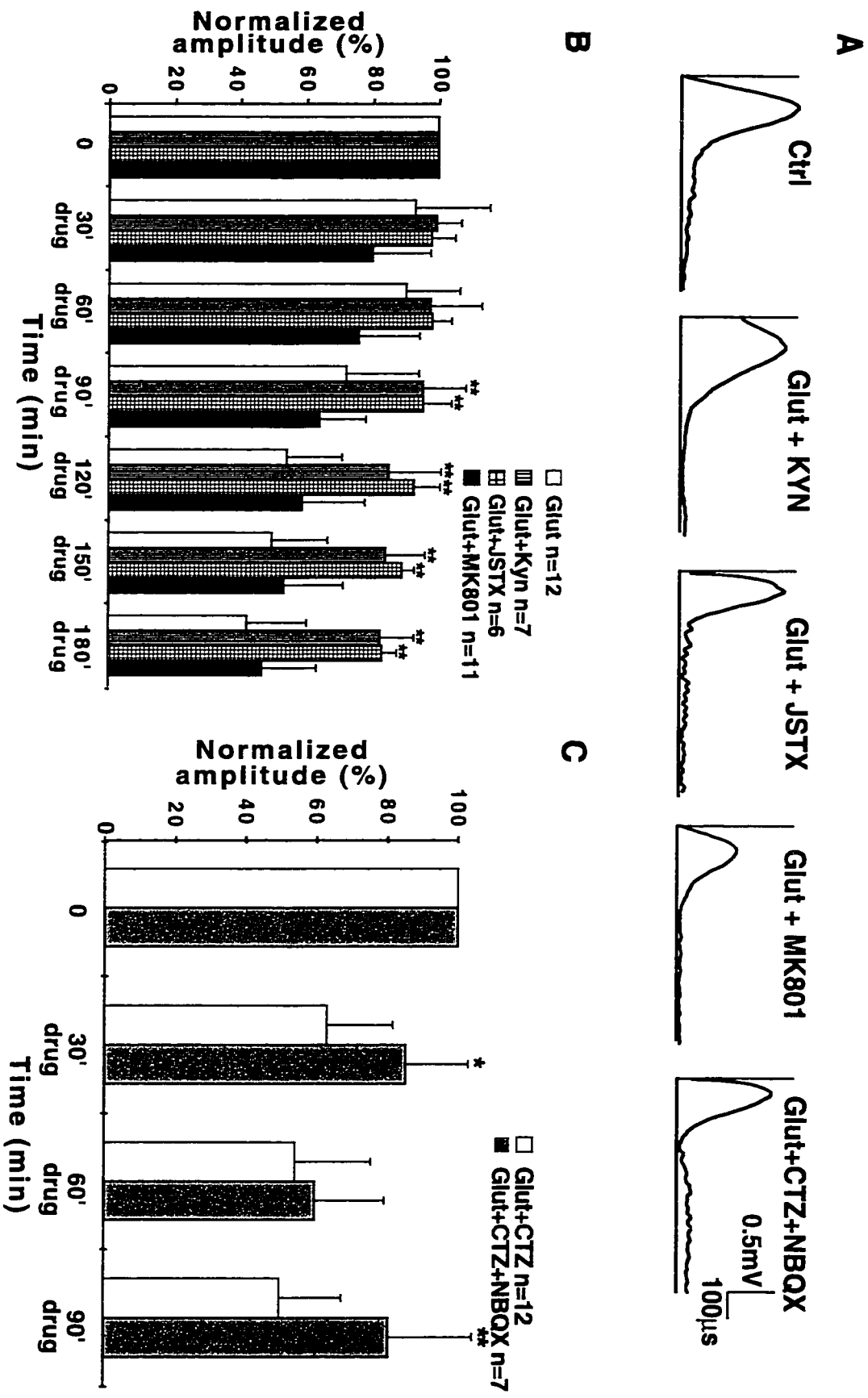


Figure 3

Figure 4 Protective effects of zero-Ca²⁺ perfusate and selective AMPA receptor blockade against kainate toxicity.

(A) Representative CAP tracings after a 180 min exposure to kainate (500 μ M), kainate in zero-Ca²⁺ (+ 100 μ M EGTA) or kainate + GYKI52466 (30 μ M).

(B) Bar graph of normalized mean CAP amplitudes showing significant protection against kainate toxicity by removal of Ca²⁺ from the perfusate or application of the selective AMPA receptor antagonist GYKI52466. These data, together with those of Fig. 3, indicate that dorsal column excitotoxicity is largely dependent on influx of extracellular Ca²⁺ triggered by activation of AMPA receptors. *P<0.05, **P<0.01 compared to time-matched readings in 500 μ M kainate and normal [Ca²⁺].

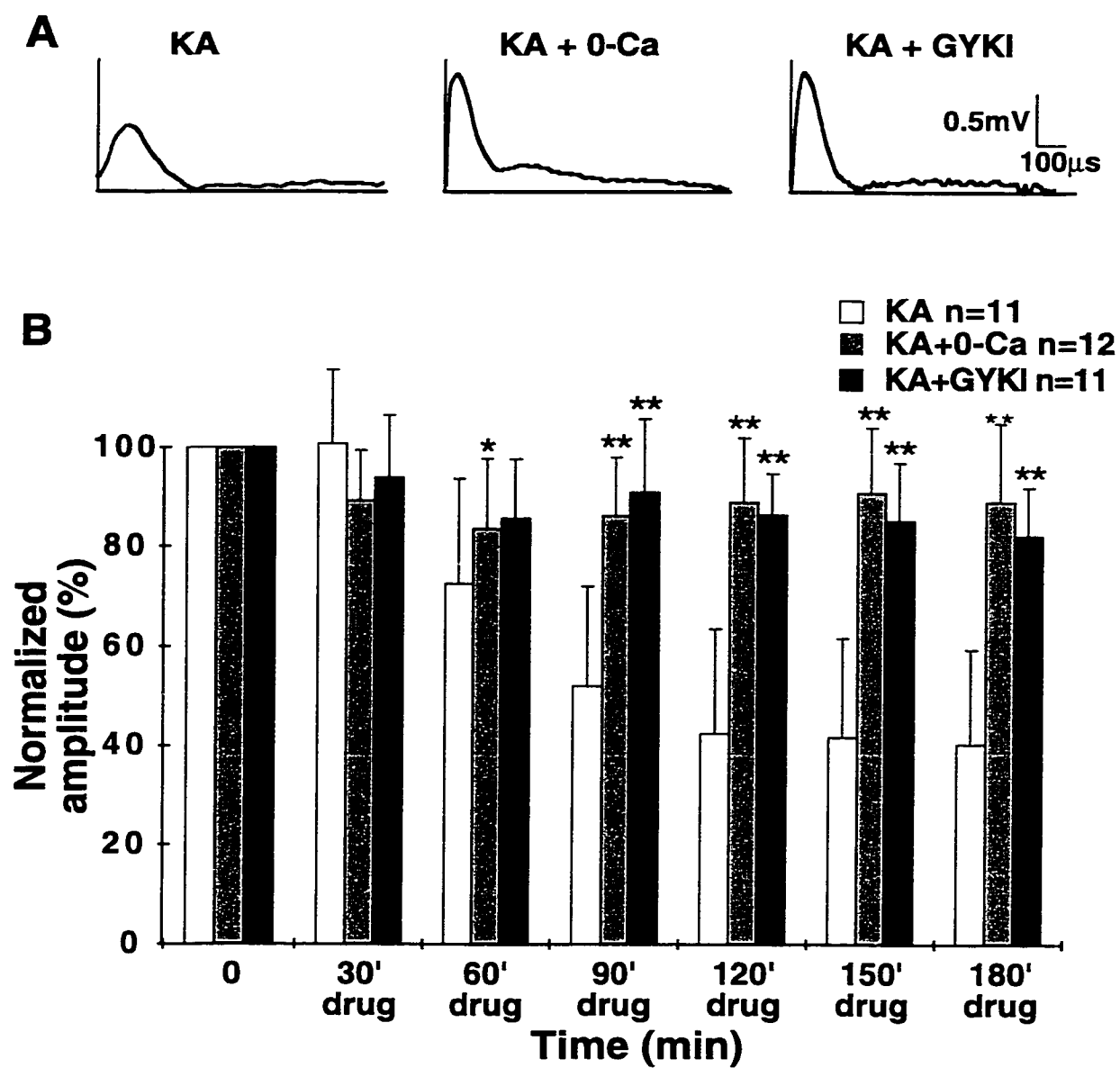


Figure 4

Figure 5 Confocal microscopic images of dorsal columns stained immunohistochemically with standard markers (neurofilament, CNPase, GFAP to identify axon cylinders, oligodendrocytes and astrocytes, respectively) and markers of cellular injury after a 3 h *in vitro* incubation in normal CSF ('Ctrl' image column) or 1 mM glutamate ('Glut'). Panels A, B and C show tissue double stained for neurofilament (green) and degenerated myelin basic protein (red, see text). Control images show no myelin damage, whereas exposure to glutamate caused marked injury to the myelin sheath surrounding most axon cylinders (arrowheads). Panels D and E identify oligodendrocytes using CNPase staining (green), showing cytoskeletal damage demonstrated by a marked increase in spectrin breakdown products ('SBP', red) in glutamate-treated vs. control slices. GFAP (green in panels F and G) identifies astrocytes that also sustained cytoskeletal damage as shown by increased spectrin breakdown (red) in cells exposed to glutamate. In contrast, axon cylinders showed no appreciable increase in spectrin breakdown products after a 3 h glutamate treatment (panels H and I). Scale bars 10 μ m.

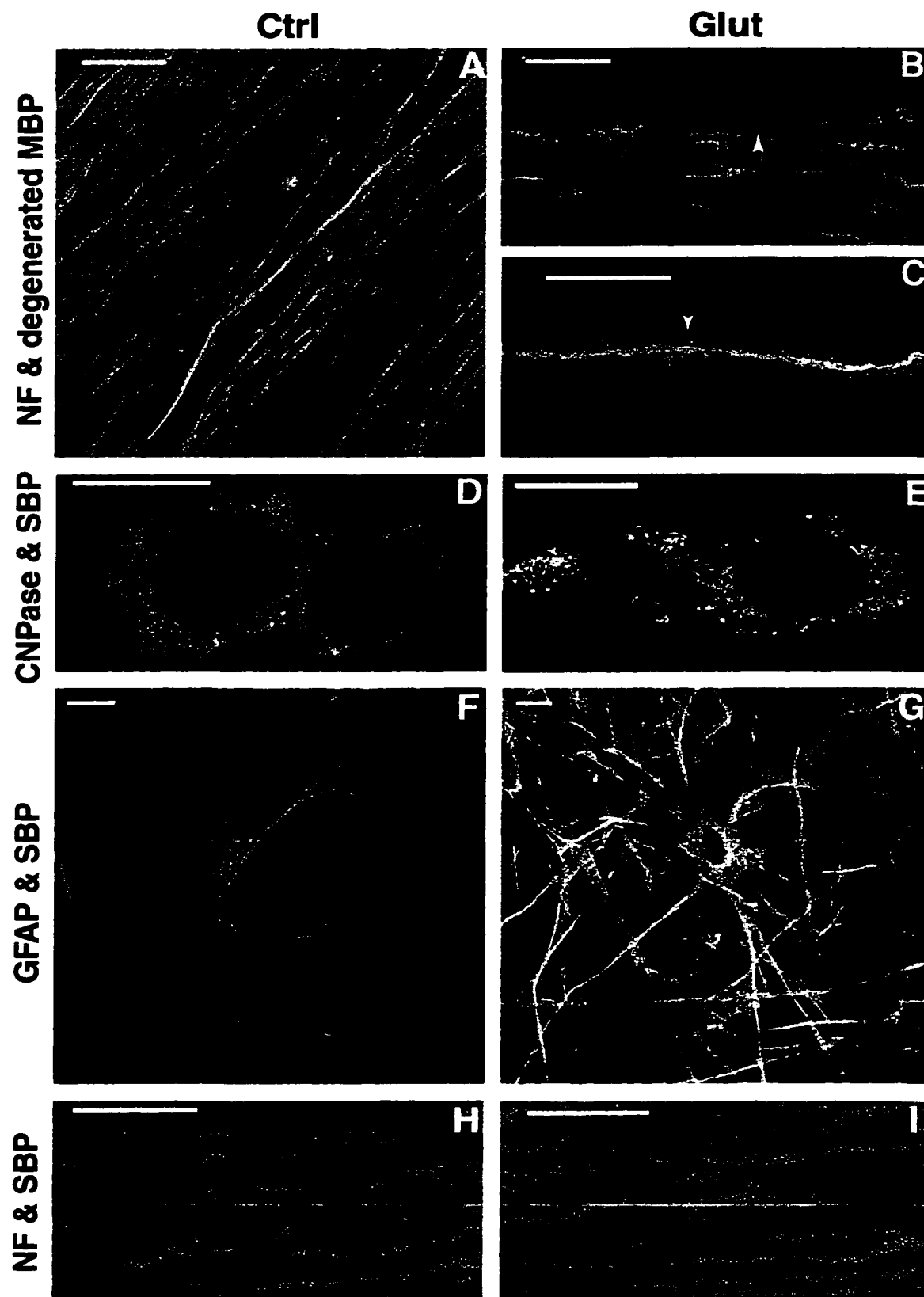


Figure 5

Figure 6 Quantitative estimates of structural injury in dorsal columns exposed to 3 h of 1 mM glutamate *in vitro*. Immunohistochemistry showing separated gray scale images (e.g. panels A and C are two channels from the same image) of sections double stained with standard markers (neurofilament, CNPase and GFAP) and spectrin breakdown products, in control and glutamate-treated tissue. Regions of interest outlined by each of the standard markers were analyzed for fluorescence intensity from the spectrin breakdown channel, thus producing a semi-quantitative estimate of spectrin degradation in oligodendrocytes, axon cylinders and astrocytes. Mean spectrin breakdown fluorescence from each of these three white matter elements is plotted in the bar graphs. Control sections show detectable levels of spectrin breakdown in all three cell types (panels C, G, K and bar graphs). Glutamate significantly increased the levels of spectrin degradation in oligodendrocytes and especially astrocytes (panels D and L), but had no effect on axon cylinders (compare panels G and H; middle bar graph). n's represent number of image pairs analyzed for each bar. Scale bars 10 μ m.

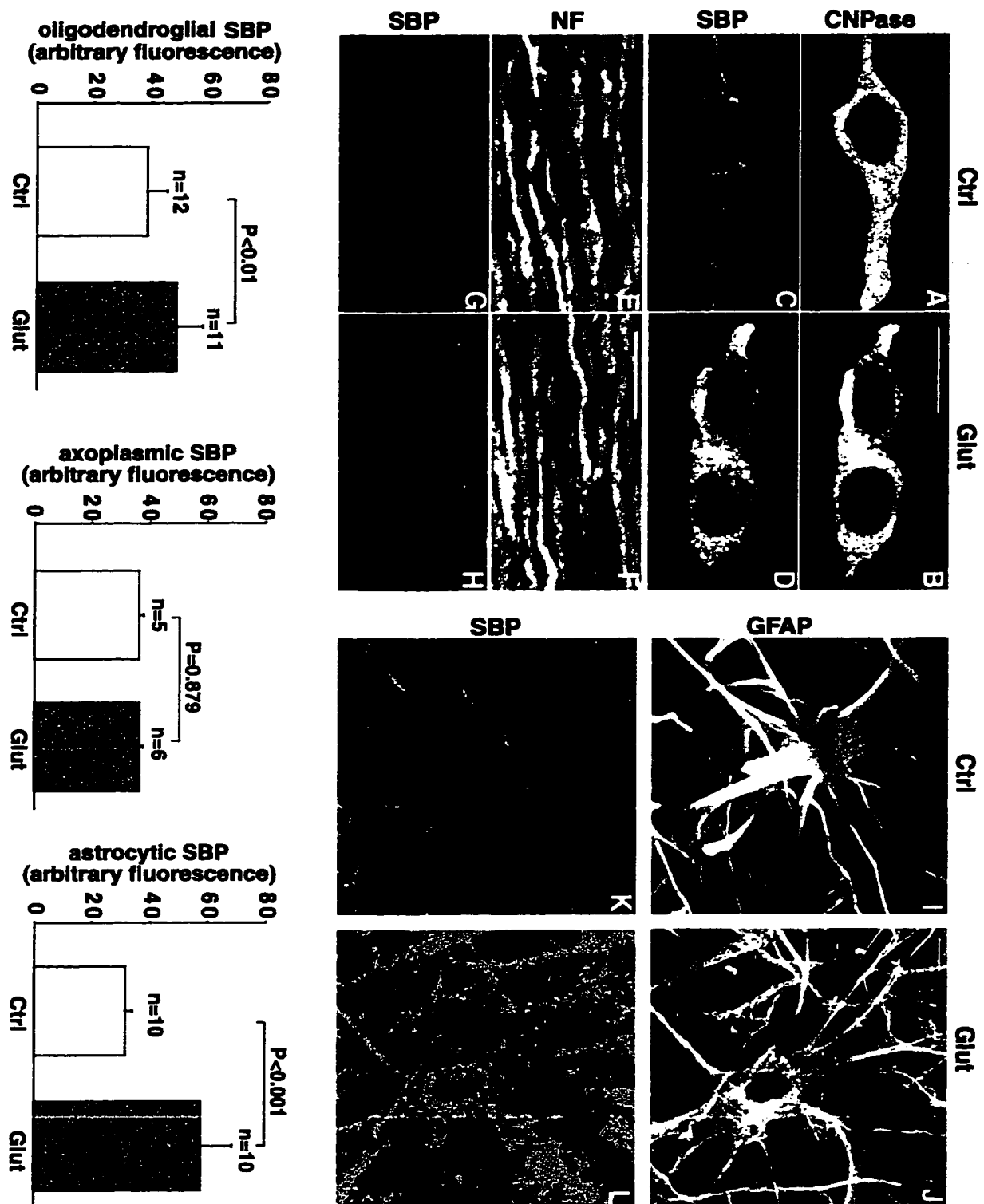


Figure 6

Figure 7 Immunohistochemical localization of AMPA receptor subunits in dorsal columns. Tissue was double stained with GluR1, GluR2/3 or GluR4 (red) and a standard marker (green). GluR2 staining was consistently absent in dorsal column white matter (not shown).

(A) Control section with primary antibody omitted.

(B) GluR1 was only faintly detected in astrocytes as shown by the yellow hue indicating colocalization of the GluR1 and GFAP signals.

(C) (D) Moderate levels of GluR2/3 were observed in oligodendrocytes and astrocytes, respectively, representing GluR3 given that GluR2 was absent.

(E) Myelin basic protein label (green) outlines myelinated dorsal column axons whose axoplasm displays strong immunoreactivity to GluR4.

GluR4 was also detected in astrocytes (F) and oligodendrocytes (G).

(H) Neurofilament stain (green) delineates an axon cylinder which is surrounded by easily detectable GluR4 signal (red) in the myelin sheath (arrow). A GluR4-positive oligodendrocyte is seen adjacent to this fiber.

Scale bars 10 μ m.

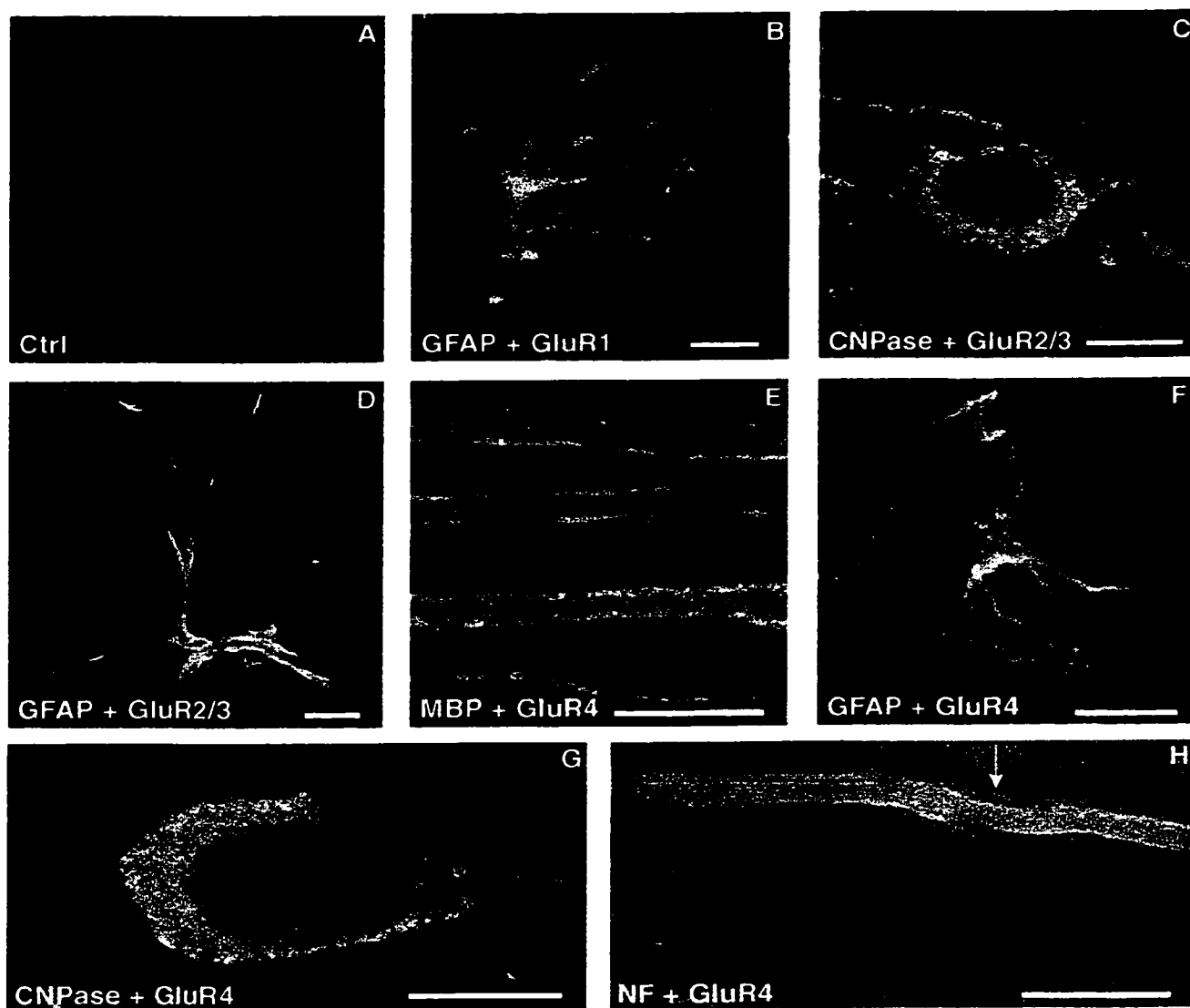


Figure 7

Chapter 3

Ouabain plus High K^+ Depolarization Induces Glutamate Release via Reverse Na^+ - Dependent Transport in Spinal Cord White Matter

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ABSTRACT

Excitotoxic mechanisms involving AMPA/kainate receptors play an important role in mediating cellular damage in spinal cord injury. However, the precise cellular mechanisms of glutamate release from non-synaptic white matter are not well understood. In the present study, we tested whether collapse or reversal of transmembrane Na^+ and K^+ gradients would reverse the operation of high affinity Na^+ -dependent transporters, inducing glutamate efflux and tissue damage by activating ionotropic glutamate receptors. Using an *in vitro* model of isolated spinal dorsal columns at 37°C, compound action potentials (CAPs) were irreversibly reduced to 42% of control after 30 min of ouabain (500 μM) (to increase $[\text{Na}^+]_i$) followed by 1 hr ouabain + high $[\text{K}^+]_o$ (129 mM/27 $[\text{Na}^+]_o$, equimolar replacement). The broad spectrum glutamate antagonist kynurenic acid (1 mM) or the selective AMPA antagonist GYKI52466 (30 μM) were partially protective in this paradigm (CAP amplitude recovered to 68%). Ca^{2+} -free perfusate was even more protective (87%). Inhibition of Na^+ -dependent glutamate transport with L-trans-pyrrolidine-2,4-dicarboxylic acid (trans-PDC, 1 mM) provided significant protection (71%), similar to that seen with glutamate receptor antagonists. Semiquantitative glutamate immunohistochemistry revealed that levels of this amino acid were significantly depleted in axon cylinders and, to a lesser degree, in oligodendrocytes (but not in astrocytes) by ouabain + high K^+ , which was largely prevented by trans-PDC. These findings support that intracellular Na^+ accumulation and depolarization drive the Na^+ -dependent glutamate transporter to operate in a reverse mode, resulting in glutamate release principally from axoplasm, and inducing spinal cord white matter injury by

activation of AMPA receptors. This mechanism may play an important role during ischemic and traumatic white matter injury, in which similar ion alterations occur.

Key words: excitotoxicity, AMPA receptor, glutamate transporter, calcium, axonal injury, GYKI52466, L-trans-pyrrolidine-2,4-dicarboxylic acid

INTRODUCTION

Oligodendrocytes and astrocytes have been shown to possess ionotropic glutamate receptors of the AMPA and kainate subtypes (Agrawal and Fehlings, 1997; Garcia-Barcina and Matute, 1996; Jensen and Chiu, 1993; Matute and Miledi, 1993; Matute et al., 1997; Steinhauser and Gallo, 1996). Persistent activation of these receptors may cause injury to oligodendrocytes both in cell culture and *in vivo* (Matute, 1998; Matute et al., 1997; McDonald et al., 1998; Yoshioka et al., 1996; Yoshioka et al., 1995). Similarly, overactivation of AMPA receptors is very toxic and even lethal to astrocytes when receptor desensitization is blocked (David et al., 1996). Recently, using isolated rat spinal cord dorsal column slices, we have characterized the pharmacological features of excitotoxic injury in an *in vitro* spinal cord white matter model at physiological temperature, and found that spinal cord white matter is markedly damaged by activation of AMPA receptors (Li and Stys, 2000). We have also localized the subcellular loci of injury, and found that oligodendrocytes, astrocytes and, in particular, the myelin sheath, are the targets for excitotoxic injury, with little evidence of damage to axon cylinders *per se*.

Using *in vitro* and *in vivo* spinal cord injury (SCI) models, several groups have shown that excitotoxic mechanisms involving AMPA/kainate receptors play an important role in mediating cellular injury in SCI (Agrawal and Fehlings, 1997; Li et al., 1999; Rosenberg et al., 1999; Teng and Wrathall, 1997; Wrathall et al., 1994). However, the precise cellular mechanisms of glutamate release from white matter tissue, devoid of synaptic elements, are not well understood. High affinity Na⁺-dependent glutamate transporters,

which have been localized in white matter (Choi and Chiu, 1997; Domercq et al., 1999; Li et al., 1999), are electrogenic with a unique ion coupling pattern, utilizing Na^+ and K^+ gradients to power glutamate uptake. Thus, alterations of Na^+ and K^+ gradients, such as elevated extracellular K^+ and internal Na^+ concentrations, together with changes in membrane potential, may affect the rate and direction of glutamate transport (Szatkowski and Attwell, 1994; Szatkowski et al., 1990; Takahashi et al., 1997). In this study, using isolated spinal cord white matter slices, we demonstrate that endogenous glutamate can be released by reversal of Na^+ -dependent glutamate transport induced by collapsing Na^+ and K^+ gradients across the plasma membrane. We also show that axonal cylinders, which contain high concentrations of this amino acid, are the major source of glutamate release, though oligodendroglia are also shown to release this amino acid to a lesser extent. Our findings would explain the observations that both Na^+ channel blockade and ionotropic glutamate antagonism are neuroprotective in SCI, as Na^+ entry through voltage-gated Na^+ channels would induce reverse glutamate transport and cause release of potentially large amounts of this excitotoxin from intracellular compartments (Taylor et al., 1995).

MATERIALS AND METHODS

Electrophysiology. Experimental procedures have been previously described (Li and Stys, 2000). Briefly, adult Long-Evans male rats (200-250 g) were deeply anesthetized with sodium pentobarbital, and a thoracic laminectomy performed. Rats were then perfused intra-aortically with 500 ml zero- Na^+ / zero- Ca^{2+} solution (in mM: choline chloride: 135; choline bicarbonate: 26; KCl: 1; KH_2PO_4 : 1.2; dextrose: 10 and EGTA:

1.0, bubbled with 95% O₂ / 5%CO₂). A 30 mm section of spinal cord was rapidly removed and placed in cold (4–6 °C) zero-Na⁺ / zero-Ca²⁺ solution bubbled with 95% O₂ / 5%CO₂. The spinal cord section was hemisected, the dorsal columns gently excised and placed in an interface recording chamber bathed in normal-Na⁺ / zero-Ca²⁺ solution (mM: NaCl: 126, KCl: 3.0, MgSO₄: 2.0, NaHCO₃: 26, NaH₂PO₄ 1.25, MgCl₂: 2.0, dextrose: 10, EGTA: 0.5 mM) at room temperature bubbled with 95% O₂ / 5%CO₂. The bath temperature was slowly raised to and maintained at 37 °C with a temperature controller (Model TC-102, Medical Systems Corp, Greenvale, NY), then the perfusate was switched to artificial cerebrospinal fluid containing normal [Ca²⁺] ('aCSF', in mM: NaCl: 126, KCl: 3.0, MgSO₄: 2.0, NaHCO₃: 26, NaH₂PO₄ 1.25, CaCl₂: 2.0, dextrose: 10). Control recordings were taken 30 min after the temperature reached 37 °C in aCSF.

Propagated compound action potentials (CAPs) were evoked using a bipolar silver wire stimulating electrode placed on one end of the dorsal column and delivering a constant voltage pulse (50 µs and typically 70 V). CAPs were recorded extracellularly at the opposite end using large-tipped glass microelectrodes filled with 150mM NaCl. To allow recording of multiple slices during a single experiment, the stimulation and recording sites were marked with a small amount of neutral red dye to allow accurate repositioning of the electrodes. Evoked CAPs were digitized, stored and analyzed using WaveTrak software without rectification (Stys, 1994). The functional integrity of the dorsal column was quantitated by measuring peak CAP amplitude.

Pharmacological agents and Protocol. Ouabain (Tocris), kynurenic acid (RBI) were directly dissolved in aCSF or high- K^+ / low- Na^+ solution (in mM: KCl: 129, $MgSO_4$: 2.0, $NaHCO_3$: 26, NaH_2PO_4 1.25, $CaCl_2$: 2.0, dextrose: 10). GYKI52466 (RBI), L-trans-pyrrolidine-2,4-dicarboxylic acid (trans-PDC, Tocris) and KB-R7943 (2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl] isothiourrea methanesulfonate) (a generous gift from Kanebo Ltd., Osaka, Japan) were first dissolved in 0.1N HCl (GYKI52466) or 0.1N NaOH (trans-PDC) or dimethylsulphoxide (KB-R7943), then added to aCSF or high- K^+ / low- Na^+ solution to the desired final concentrations. The pH of the solutions was maintained at 7.4. Glutamate receptor antagonists (kynurenic acid, GYKI52466), and the inhibitors of glutamate transport (trans-PDC) or Na^+ - Ca^{2+} exchange (KB-R7943) were applied beginning 30 min before addition of ouabain, and continued until 30 min after ouabain (500 μ M) + high K^+ perfusion (129 mM).

Immunohistochemistry of glutamate. We used quantitative confocal immunofluorescence (Li et al., 1999) to directly examine whether disruption of Na^+ and K^+ gradients would deplete glutamate in intracellular compartments of dorsal column white matter, and whether inhibition of Na^+ -dependent glutamate transport with trans-PDC would prevent glutamate release from intracellular space. Following surgical preparation and spinal cord dissection as above, dorsal column slices were incubated in: a). normal aCSF for 2 hrs (normal Ctrl); b). 30 min aCSF + 30 min 500 μ M ouabain + 60 min 500 μ M ouabain/high K^+ (129 mM) (ouabain Ctrl); c). b) + 1 mM trans-PDC. Then, these slices were fixed in 4% paraformaldehyde + 0.5% glutaraldehyde for 24 hours, and cryoprotected for 48 hrs in phosphate-buffered saline (PBS, pH7.4) containing 20%

glycerol at 4 °C. Slices were then dissected into smaller pieces (approximately 3 x 1.5 x 0.5 mm), preincubated in 10% Triton X-100 for 30 min, followed by 4% normal goat serum (NGS) with 0.1% Triton X-100, and PBS for blocking for 1 hr at room temperature. After a single quick rinse in PBS, the sections were incubated for 24 hours at 4 °C with rabbit anti-glutamate polyclonal antibody (Chemicon) at 1:500 diluted in 2% NGS with 0.1% Triton X-100, PBS. Double staining with monoclonals against neurofilament 160 (Sigma), 2',3'-cyclic-nucleotide 3'-phosphodiesterase ('CNPase', Chemicon), anti-glial fibrillary acidic protein ('GFAP', Boehringer Mannheim) was performed to localize axon cylinders, oligodendrocytes (Trapp et al., 1988) and astrocytes, respectively. Following the primary antibody incubation, slices were rinsed three times in PBS for 30 minutes, then incubated for 1 hr with Alexa™ 594 goat anti-rabbit (1:200) and Alexa™ 488 goat anti-mouse (1:400, Molecular Probes) diluted in PBS with 2% NGS and 0.1% Triton X-100. Control sections were processed with the same procedures except the omission of either the primary antisera or secondary antibodies. Images were collected on a Bio-Rad 1024 confocal laser scanning microscope with a 60X oil-immersion objective (Olympus). A minimum of 6 images collected from each of 2 to 3 sections were examined for each marker from each of 3 groups, and representative images are shown. The digitized images were analyzed blindly using NIH Image 1.61 (<http://rsb.info.nih.gov/nih-image/default.html>) on a Macintosh Power PC.

Statistics. All data are expressed as means $\pm$ standard deviation. Statistical differences were calculated by ANOVA with Dunnett's test for multiple comparisons with a common control group in the case of electrophysiological data. Student's t test was used for

quantitative immunofluorescence data when only two groups were compared. Reported n's represent number of individual dorsal column slices studied electrophysiologically, or the number of confocal image frames analyzed for fluorescence intensity.

RESULTS

Deregulation of ion gradients by ouabain plus high K⁺ injures dorsal column white matter

Electrophysiological recording of peak CAP amplitude remained stable in control dorsal column slices perfused with normal aCSF for more than 3 hrs (Fig.1). Slices incubated for 30 min in the selective Na⁺-K⁺-ATPase inhibitor ouabain (500 μ M) exhibited decreased CAP amplitude ($23 \pm 9\%$ of control). Following 60 min of 500 μ M ouabain + high K⁺ (129 mM, equimolar ion replacement), the peak CAP amplitude was further attenuated to a mean value of $\approx 4\%$ (CAPs absent in the majority of slices). CAP amplitude recovered to $\approx 43\%$ of pre-drug control after 2 hr wash with aCSF, indicating irreversible impairment of dorsal column axonal function induced by pump blockade and depolarization. Because intracellular Ca²⁺ overload is a key step in the series of events leading to injury under a variety of pathologic conditions (Kristian and Siesjo, 1998; Stys, 1998; Tymianski and Tator, 1996) and internal Ca²⁺ accumulation is the “final common pathway” of neural cell death (Nicotera et al., 1992; Siesjö, 1986), the role of external Ca²⁺ in axonal injury was examined in this experimental paradigm. Compared with time-matched ouabain + high K⁺ control, the recovery of CAP amplitude was significantly improved to $\approx 87\%$ of control by removing Ca²⁺ from the perfusate,

indicating that functional damage of dorsal column white matter as a result of reduced Na^+ and K^+ gradients is largely dependent on extracellular Ca^{2+} .

AMPA receptors contribute to axonal damage induced by ionic disruptions

Loss of electrical excitability as a result of collapse of Na^+ and K^+ gradients itself is usually not enough to induce irreversible injury to myelinated axons (LoPachin and Stys, 1995; Stys, 1998), suggesting the involvement of other cellular mechanisms in the irreversible injury of dorsal column slices in present paradigm. Recent studies indicate that glutamate toxicity markedly contributes to white matter injury depending on overactivation of non-NMDA receptors present on glial cells (Agrawal and Fehlings, 1997; Li and Stys, 2000; Wrathall et al., 1994). To evaluate the possible role of excitatory amino acids, the effects of ionotropic glutamate receptor antagonists on the recovery of compound action potentials following inhibition of Na^+ - K^+ -ATPase and high K^+ perfusion were measured. As indicated by the significant improvement of CAP amplitude in Fig.2, both the broad spectrum glutamate receptor antagonist kynurenic acid (1 mM) and selective AMPA receptor antagonist GYKI52466 (30 μM) (Paternain et al., 1995) were significantly neuroprotective against white matter injury induced by Na^+ - K^+ -ATPase inhibition and high K^+ exposure. These data illustrate that endogenous glutamate contributes to dorsal column axonal injury induced by collapse of transmembrane Na^+ and K^+ gradients, and that this toxicity is mediated mainly via ionotropic glutamate receptors of the AMPA subtype.

Glutamate is released via reverse Na^+ -dependent glutamate transport

In the absence of membrane rupture, endogenous glutamate could be released by several pathways: Ca^{2+} -dependent vesicular release, Ca^{2+} -independent efflux through swelling-activated anion channels (Basarsky et al., 1999) or by reverse operation of glutamate transporters (Attwell et al., 1993; Szatkowski et al., 1990). In the present experiments with isolated dorsal column white matter, a preparation without synaptic machinery, the endogenous excitatory amino acid must be released by a Ca^{2+} -independent route(s). To test whether glutamate is released via the Na^+ -dependent transporter, the effects of glutamate transport inhibitor on the recovery of CAP amplitude were investigated during inhibition of Na^+ - K^+ -ATPase and high K^+ application. As shown in Fig.3, L-trans-pyrrolidine-2,4-dicarboxylic acid (1 mM), a transportable inhibitor of Na^+ -dependent glutamate transport, provided significant neuroprotection to dorsal column slices exposed to ouabain (500 μM) and high K^+ milieu as this agent improved CAP amplitude to $\approx 72\%$ (vs. $\approx 43\%$ without transport inhibitor). This finding supports the notion that decreased or reverse transmembrane Na^+ and K^+ gradients may induce internal glutamate release through reverse operation of Na^+ -dependent glutamate transport, subsequently resulting in conduction impairment of dorsal column white matter. However, inhibition of Na^+ - Ca^{2+} exchanger with KB-R7943 did not have significant effects on CAP amplitude recovery (Fig. 3 C).

Axon cylinders are the main source of endogenous glutamate

The studies of combining electrophysiology and pharmacology suggest collapses of Na^+ and K^+ gradients cause glutamate efflux from an intracellular compartment. To further confirm this notion and to identify which cell components are capable of releasing this

amino acid, semiquantitative glutamate immunohistochemistry in isolated dorsal column slices was performed using double staining for standard markers (neurofilament, CNPase and GFAP) and glutamate (panel A-H in Fig. 4). Mean glutamate fluorescence measured from axon cylinders, oligodendroglia and astrocytes using confocal microscopy was analyzed in 3 groups: normal control, ouabain + high K^+ without and with treatment with glutamate transport inhibitor. As shown by the bar graph in Fig.4, the glutamate signal was significantly attenuated in both axon cylinders and oligodendrocytes, particularly in the former, following exposure to ouabain (500 μ M) and high K^+ (129 mM, equimolar ion replacement). However, treatment with glutamate transport inhibitor L-trans-pyrrolidine-2,4-dicarboxylic acid (1 mM) almost completely prevented the attenuation of glutamate fluorescence from axon cylinders (black bar on left), though this agent did not statistically prevent glutamate release from oligodendrocytes, possibly due to efflux of this amino acid partly through other routes such as swelling-activated anion channels (Basarsky et al., 1999). In contrast, glutamate fluorescence in astrocytes did not change significantly compared with time-matched slices incubated in aCSF without any drugs. These results confirm the idea that glutamate efflux is mainly via reverse Na^+ -dependent glutamate transport, and more importantly, demonstrate that the release of glutamate is principally from axon cylinders, and to a lesser degree from oligodendrocytes, during collapse of Na^+ and K^+ gradients, but not from astrocytes.

DISCUSSION

Na^+ -dependent glutamate transporters provide the majority of glutamate uptake and are essential for maintaining low extracellular glutamate and for preventing glutamate

toxicity (Peghini et al., 1997; Rothstein et al., 1996; Tanaka et al., 1997). For example, at glutamatergic synapses, an essential component of transmission is to remove glutamate from the synaptic clefts. The transporters situated on presynaptic terminals or the surrounding glia efficiently sequester glutamate from the synaptic cleft. To date, 5 subtypes of Na⁺-dependent glutamate transporters have been identified in the nervous system: GLAST (EAAT1), GLT1 (EAAT2), EAAC1 (EAAT3), EAAT4 and EAAT5 (for review see Gegelashvili and Schousboe, 1998; Vandenberg, 1998). The affinity of each subtype of transporter for excitatory amino acids is different, indicating possible functional specialization of these isoforms. GLT1, with a low K_m (~2 μM) for L-glutamate, appears to play an essential role in maintaining [Glu]_o at low concentration. EAAC1, a neuronal glutamate transporter with a K_m of ~12 μM, possibly functions as a presynaptic glutamate uptake carrier. In contrast, GLAST, with a high K_m for glutamate (~70 μM), might be a reserve to prevent [Glu]_o from reaching toxic levels. Common to all these transporters is the coupling to cotransport of 3 Na⁺ ions, 1 proton (or countertransport of 1 hydroxyl ion) and countertransport of 1 K⁺ for uptake of 1 glutamate molecule into the cell (Kanai et al., 1995; Levy et al., 1998; Takahashi et al., 1997; Zerangue and Kavanaugh, 1996). The net transport of one charge per cycle renders this ion transporter electrogenic. Thus, alterations of Na⁺ and K⁺ gradients, and a membrane potential will affect the rate and direction of glutamate transport. Under normal conditions, the large transmembrane Na⁺ and K⁺ gradients and hyperpolarized membrane potential favor transport of glutamate into cells against a large gradients, maintaining low [Glu]_o levels. However, when the Na⁺ and K⁺ gradients are attenuated and membrane potential is depolarized, these transporters may be driven to operate in a

reverse mode, eliciting glutamate efflux from intracellular compartment of cells (Attwell et al., 1993; Gamba et al., 1994; Longuemare and Swanson, 1997; Rutledge and Kimelberg, 1996; Szatkowski et al., 1990; Tachibana and Okada, 1991).

During anoxia/ischemia and trauma, release of glutamate significantly contributes to cell death and clinical disability (Obrenovitch, 1996; Obrenovitch and Urenjak, 1997; Szatkowski and Attwell, 1994). The mechanism of glutamate release has been thought to be mainly from vesicular release dependent on internal Ca^{2+} rise (Drejer et al., 1985; Katayama et al., 1991). However, studies indicate that the ATP drop due to anoxia/ischemia in brain slices would inhibit the docking of vesicles and release of glutamate from synapses via a Ca^{2+} -dependent route (McMahon and Nicholls, 1991; Szatkowski and Attwell, 1994; Takahashi et al., 1997); instead, a significant fraction of glutamate is released in a Ca^{2+} -independent manner through reverse operation of Na^{+} -dependent glutamate transport (Attwell et al., 1993; Ikeda et al., 1989; Sanchez-Prieto and Gonzalez, 1988). During anoxia/ischemia, the fall of ATP levels due to oxygen deprivation would lead to the slowing of Na^{+} - K^{+} -ATPase and a rundown of the transmembrane Na^{+} and K^{+} gradients and membrane potential (Szatkowski and Attwell, 1994; Agrawal and Fehlings, 1996), which in turn would drive glutamate transport to operate in a reverse fashion and favor glutamate efflux from internal compartments (Roettger and Lipton, 1996; Rossi et al., 2000).

In CNS white matter which is devoid of synaptic structures, several subtypes (GLT1, GLAST and EAAC1) of Na^{+} -dependent glutamate transporters have been shown to be

expressed in white matter (Choi and Chiu, 1997; Domercq et al., 1999). These transporters may also be induced to run in the glutamate export mode given the reduced transmembrane Na^+ and K^+ gradients. Indeed, using 60 min anoxia and clip compression injury models of dorsal column slices, we recently provide evidence that loss of Na^+ and K^+ homeostasis (LoPachin et al., 1999) results in the release of glutamate by reversal of Na^+ -dependent transporters from intracellular compartments of myelinated axons, and the uncontrolled efflux of this excitotoxin plays an important role in anoxic/traumatic white matter injury via subsequent activation of ionotropic glutamate receptors (Li et al., 1999). In the present study, using uninjured isolated dorsal column slices, we tested whether the attenuation, or reversal of Na^+ and K^+ gradients would trigger glutamate release via reverse transport and cause irreversible functional failure of axons.

Physiologically, Na^+ - K^+ -ATPase plays a key role in maintaining transmembrane Na^+ and K^+ gradients (Erecinska and Dagani, 1990). Inhibition of this pump, which occurs frequently during a number of insults (such as anoxia/ischemia, hypoglycemia and trauma), would induce accumulation of $[\text{Na}^+]_i$ and $[\text{K}^+]_o$ by ion flux via voltage-gated Na^+/K^+ channels, neurotransmitter-activated ion channels (such as AMPA/kainate receptors) or $\text{Na}^+ + \text{K}^+ + 2\text{Cl}^-$ co-transporters. In the present experiments, we exposed dorsal spinal white matter slices to the Na^+ - K^+ -ATPase inhibitor ouabain, which would elevate axonal $[\text{Na}^+]_i$ to ≈ 120 mM (LoPachin and Stys, unpublished data). To further reverse the Na^+ and K^+ gradients and depolarize potentials across membranes, we then perfused the slices with high K^+ solution (129 mM K^+ /27 mM Na^+) co-applied with ouabain. Following this treatment, $[\text{K}^+]_i$ should be obviously reduced (~ 18 mM, given

that the ratio of $[Na^+]_i/[K^+]_i$ was raised to 6.6 from 0.15 after ouabain application) (Krep et al., 1996); the membrane potentials should be depolarized to positive voltages ($\sim +50$ mV, equilibrium potential of K^+) as a result of reverse transmembrane K^+ gradients ($[K^+]_o/[K^+]_i = 129/18$ mM). Given that the normal transmembrane gradients of Na^+ ($[Na^+]_o/[Na^+]_i = 140/17$ mM), K^+ ($[K^+]_o/[K^+]_i = 3/120$ mM) and hyperpolarized membrane potentials (-80 mV) are able to keep extracellular glutamate at very low concentrations ($\sim 1\mu M$), the apparent reverse gradients of Na^+ and K^+ together with strong membrane depolarization induced in the present experiments should provide enough backward driving power for Na^+ -dependent glutamate transporters to efflux glutamate from intracellular compartments and to deplete internal pools of this amino acid.

Using electrophysiology as an outcome measure of functional integrity, we found that inhibition of Na^+ - K^+ -ATPase combined with high K^+ exposure resulted in irreversible depression of CAP amplitude to 43% of control value. This finding is consistent with the reports that inhibition of Na^+ - K^+ -ATPase activity evoked significant neuronal damage in gray matter (Lees and Leong, 1994; Lees and Leong, 1995; Omar et al., 2000). Since alteration of Na^+ and K^+ gradients itself appears not to elicit irreversible injury to myelinated axons (LoPachin and Stys, 1995; Stys, 1998), in this study, other mechanisms, such as secondary internal Ca^{2+} accumulation, must involve the pathogenesis of axonal dysfunction during the ion disruptions caused by ouabain plus high K^+ . Our finding of apparent protection against axonal damage by removal of Ca^{2+} from the perfusate during inhibition of Na^+ - K^+ -ATPase (with CAP amplitude recovering to much greater than with Ca^{2+} replete perfusate), demonstrates that white matter injury

induced by the collapse of Na^+ and K^+ gradients is highly dependent on the influx of Ca^{2+} ions. This finding supports the notion that internal Ca^{2+} overload is an important and final common pathway of neural cell death (Kristian and Siesjo, 1998; Stys, 1998; Tymianski and Tator, 1996).

Recently, excitotoxicity has been shown to be an important mechanism in CNS axonal injury under various conditions including anoxia/trauma (Agrawal and Fehlings, 1997; Li et al., 1999; Rosenberg et al., 1999; Wrathall et al., 1994), multiple sclerosis (Pitt et al., 2000) or exogenous excitotoxin exposure (Li and Stys, 2000; Matute, 1998; Matute et al., 1997; McDonald et al., 1998). In this paradigm, we found that the broad spectrum glutamate receptor antagonist kynurenic acid or selective AMPA receptor antagonist GYKI52466 (Paternain et al., 1995) provided significantly and virtually identical neuroprotection to spinal cord white matter, suggesting that Ca^{2+} influx via ionotropic glutamate receptors, particularly AMPA receptors, plays a considerable role in white matter injury caused by depression of ion gradients across membrane. These findings are consistent with the presence of AMPA receptor subunits in dorsal column white matter and the vulnerability of CNS white matter tissues to excitotoxins (Agrawal and Fehlings, 1997; Li and Stys, 2000; Matute, 1998; McDonald et al., 1998). The functional impairment of axons caused by excitotoxins is considered to be principally attributed to the damage to glial elements (Li and Stys, 2000; Matute, 1998; McDonald et al., 1998; Rosenberg et al., 1999), particularly to the myelin sheath (Li et al., 1999; Li and Stys, 2000). The disruption of the sheath may result in slowing or even complete failure of

signal conduction along axons since this structure plays a critical role in sustaining saltatory conduction of myelinated axons (Waxman, 1992).

Our pharmacological experiments provide evidence that Ca^{2+} influx involving ionotropic glutamate receptors, primarily AMPA receptors, plays a considerable role in the white matter injury in this paradigm. But alternative Ca^{2+} entry routes appear also to play a role in Ca^{2+} overload since the protective effects of glutamate receptor blockers were not as great as those by removing Ca^{2+} from the perfusate (CAP amplitude recovery to 68% vs. 87%, respectively). For example, the changes in ion gradients and membrane depolarization may also induce Ca^{2+} entry through voltage-gated Ca^{2+} channels, reverse Na^+ - Ca^{2+} exchange (Hack and Balazs, 1995; Imaizumi et al., 1999; Liu et al., 1997) or even Ca^{2+} leakage through some channels mainly permeable to other ions (Stys, 1998). However, treatment with the relatively selective Na^+ - Ca^{2+} exchanger blocker KB-R7943 (Hoyt et al., 1998; Iwamoto et al., 1996) did not significantly improve function of axons during blockade of Na^+ - K^+ -ATPase and high K^+ perfusion (Fig. 3 C), indicating that the Na^+ - Ca^{2+} exchanger plays little, if any role in this paradigm, though this exchanger significantly contributes to anoxic axonal injury (Stys, 1998; Stys et al., 1992). The different roles of the Na^+ - Ca^{2+} exchanger in the present paradigm vs. anoxic axonal dysfunction are probably due to the discrepancies of Ca^{2+} buffering capability. For example, the Ca^{2+} buffering enzymes, such as Ca^{2+} -ATPase on the plasmalemma and ER, may be significantly compromised during anoxia due to the drop of ATP levels, probably contributing to Ca^{2+} accumulation in axoplasm; in contrast, the functions of these proteins are still maintained during Na^+ - K^+ -ATPase inhibition with ouabain. Thus, in the present

paradigm, the intracellular Na^+ accumulation and high K^+ depolarization would induce reverse operation of Na^+ - Ca^{2+} exchanger causing Ca^{2+} influx through this transporter, but the intact Ca^{2+} buffer systems likely extrude the Ca^{2+} from the axoplasm and buffer this ion into internal stores, preventing lethal amounts of Ca^{2+} accumulation and axonal injury. Taken together, during ouabain inhibition, the alternative Ca^{2+} entry routes contributing irreversible white matter damage possibly include VGCC and/or some ion channels mainly permeable to other ions, in addition to Ca^{2+} influx through ionotropic glutamate receptors.

In the present preparations, the source of endogenous glutamate must be from axons and/or glia and must not depend on synaptic release mechanism since only isolated dorsal column tracts devoid of synaptic components were applied. The marked neuroprotection by the glutamate transport inhibitor L-trans-pyrrolidine-2,4-dicarboxylic acid, which was similar to that seen with glutamate receptor antagonists, demonstrates that release of glutamate through reverse Na^+ -dependent transport is a principal route in the present experiments. Our finding is congruent with the reports showing the ionic deregulation induced by veratridine (an agent increasing Na^+ permeability), or by anoxia/ischemia, hypoglycemia or trauma may result in efflux of this amino acid from internal compartments in both white and gray matter preparations (Calo et al., 1997; Heron et al., 1995; Li et al., 1999; Longuemare and Swanson, 1997; Roettger and Lipton, 1996; Rossi et al., 2000; Santos et al., 1996; Stier et al., 1996; Westerink et al., 1989).

Taken together, our findings support that in CNS white matter, collapses of ion gradients and membrane depolarization could drive the glutamate transporter operate in a reverse mode, resulting in glutamate release and excitotoxic injury largely via activation of AMPA receptors. Further evidence from immunohistochemistry for glutamate staining confirms this hypothesis since inhibition of Na⁺-dependent glutamate transport prevented depletion of glutamate from intracellular space of dorsal column axons. More importantly, our experiments with confocal microscopy directly display that the axon cylinders and oligodendrocytes, especially the former, are the sources of endogenous glutamate release (Fig.4), whereas astrocytic glutamate does not change following ion gradient alterations, probably due to the relatively low glutamate concentrations in this cell type (Attwell et al., 1993). These findings are almost identical to those in our recent dorsal column study using an anoxic injury model (Li et al., 1999), and consistent with the recent report on ischemic oligodendroglial death mediated by excitotoxin released through a similar mechanism (Fern and Moller, 2000). The present results are also supported by the expression of Na⁺-dependent glutamate transporters (EAAC1, GLAST and GLT1) in CNS white matter tracts (Choi and Chiu, 1997; Li et al., 1999; Sutherland et al., 1996). Our findings help explain the neuroprotective effects of glutamate receptor antagonists against traumatic and anoxic spinal cord injury (Agrawal and Fehlings, 1997; Li et al., 1999; Rosenberg et al., 1999; Wrathall et al., 1994), during which similar ion deregulations occur and would induce endogenous glutamate release via the same mechanism as reported in this study. Our results also suggest that blocking excitotoxin release via inhibition of Na⁺-dependent transport could be an alternative approach to

efficiently prevent glutamate toxicity during the pathogenesis of such axonal insults as anoxic/ischemic and traumatic injury.

REFERENCES

Agrawal SK, Fehlings MG (1996) Mechanisms of secondary injury to spinal cord axons in vitro - role of Na^+ , $\text{Na}^+\text{-K}^+\text{-ATPase}$, the $\text{Na}^+\text{-H}^+$ exchanger, and the $\text{Na}^+\text{-Ca}^{2+}$ exchanger. *J Neurosci* 16: 545-552.

Agrawal SK, Fehlings MG (1997) Role of NMDA and non-NMDA ionotropic glutamate receptors in traumatic spinal cord axonal injury. *J Neurosci* 17: 1055-1063.

Attwell D, Barbour B, Szatkowski M (1993) Nonvesicular release of neurotransmitter. *Neuron* 11: 401-7.

Basarsky TA, Feighan D, MacVicar BA (1999) Glutamate release through volume-activated channels during spreading depression. *J Neurosci* 19: 6439-45.

Calo G, Sbrenna S, Bianchi C, Beani L (1997) Immediate and delayed effects of in vitro ischemia on glutamate efflux from guinea-pig cerebral cortex slices. *Brain Res* 751: 300-6.

Choi I, Chiu SY (1997) Expression of high-affinity neuronal and glial glutamate transporters in the rat optic nerve. *Glia* 20: 184-92.

David JC, Yamada KA, Bagwe MR, Goldberg MP (1996) AMPA receptor activation is rapidly toxic to cortical astrocytes when desensitization is blocked. *J Neurosci* 16: 200-9.

Domercq M, Sanchez-Gomez MV, Areso P, Matute C (1999) Expression of glutamate transporters in rat optic nerve oligodendrocytes. *Eur J Neurosci* 11: 2226-36.

Drejer J, Benveniste H, Diemer NH, Schousboe A (1985) Cellular origin of ischemia-induced glutamate release from brain tissue in vivo and in vitro. *J Neurochem* 45: 145-51.

Erecinska M, Dagani F (1990) Relationships between the neuronal sodium/potassium pump and energy metabolism. Effects of K^+ , Na^+ , and adenosine triphosphate in isolated brain synaptosomes. *J Gen Physiol* 95: 591-616.

Fern R, Moller T (2000) Rapid ischemic cell death in immature oligodendrocytes: A fatal glutamate release feedback loop. *J Neurosci* 20: 34-42.

Garcia-Barcina JM, Matute C (1996) Expression of kainate-selective glutamate receptor subunits in glial cells of the adult bovine white matter. *Eur J Neurosci* 8: 2379-2387.

Gegelashvili G, Schousboe A (1998) Cellular distribution and kinetic properties of high-affinity glutamate transporters. *Brain Res Bull* 45: 233-8.

Gemba T, Oshima T, Ninomiya M (1994) Glutamate efflux via the reversal of the sodium-dependent glutamate transporter caused by glycolytic inhibition in rat cultured astrocytes. *Neurosci* 63: 789-95.

Hack N, Balazs R (1995) Properties of AMPA receptors expressed in rat cerebellar granule cell cultures: Ca^{2+} influx studies. *J Neurochem* 65: 1077-84.

Heron A, Springhetti V, Seylaz J, Lasbennes F (1995) Effects of a glutamate uptake inhibitor on glutamate release induced by veratridine and ischemia. *Neurochem Int* 26: 593-9.

Hoyt KR, Arden SR, Aizenman E, Reynolds IJ (1998) Reverse $\text{Na}^+/\text{Ca}^{2+}$ exchange contributes to glutamate-induced intracellular Ca^{2+} concentration increases in cultured rat forebrain neurons. *Mol Pharmacol* 53: 742-9.

Ikeda M, Nakazawa T, Abe K, Kaneko T, Yamatsu K (1989) Extracellular accumulation of glutamate in the hippocampus induced by ischemia is not calcium dependent--in vitro and in vivo evidence. *Neurosci Lett* 96: 202-6.

Imaizumi T, Kocsis JD, Waxman SG (1999) The role of voltage-gated Ca^{2+} channels in anoxic injury of spinal cord white matter. *Brain Res* 817: 84-92.

Iwamoto T, Watano T, Shigekawa M (1996) A novel isothiourea derivative selectively inhibits the reverse mode of $\text{Na}^+/\text{Ca}^{2+}$ exchange in cells expressing NCX1. *J Biol. Chem.* 271: 22391-22397.

Jensen AM, Chiu SY (1993) Expression of glutamate receptor genes in white matter: developing and adult rat optic nerve. *J Neurosci* 13: 1664-75.

Kanai Y, Nussberger S, Romero MF, Boron WF, Hebert SC, Hediger MA (1995) Electrogenic properties of the epithelial and neuronal high affinity glutamate transporter. *J Biol Chem* 270: 16561-8.

Katayama Y, Kawamata T, Tamura T, Hovda DA, Becker DP, Tsubokawa T (1991) Calcium-dependent glutamate release concomitant with massive potassium flux during cerebral ischemia in vivo. *Brain Res* 558: 136-40.

Krep H, Lefurgey A, Graves SW, Hockett D, Ingram P, Hollenberg NK (1996) Elemental composition of Na^+ pump inhibited rabbit aorta VSM cells by electron probe X-ray microanalysis. *Am J Physiol* 271: H514-20.

Kristian T, Siesjo BK (1998) Calcium in ischemic cell death. *Stroke* 29: 705-718.

Lees GJ, Leong W (1994) Brain lesions induced by specific and non-specific inhibitors of sodium- potassium ATPase. *Brain Res* 649: 225-33.

Lees GJ, Leong W (1995) The sodium-potassium ATPase inhibitor ouabain is neurotoxic in the rat substantia nigra and striatum. *Neurosci Lett* 188: 113-6.

Levy LM, Warr O, Attwell D (1998) Stoichiometry of the glial glutamate transporter GLT-1 expressed inducibly in a chinese hamster ovary cell line selected for low endogenous Na⁺-dependent glutamate uptake. *J Neurosci* 18: 9620-8.

Li S, Mealing GA, Morley P, Stys PK (1999) Novel injury mechanism in anoxia and trauma of spinal cord white matter: glutamate release via reverse Na⁺-dependent glutamate transport. *J Neurosci* 19: RC16 (1-9).

Li S, Stys PK (2000) Mechanisms of Ionotropic Glutamate Receptor-Mediated Excitotoxicity in Isolated Spinal Cord White Matter. *J Neurosci* 20: 1190-1198.

Liu HN, Molina-Holgado E, Almazan G (1997) Glutamate-stimulated production of inositol phosphates is mediated by Ca²⁺ influx in oligodendrocyte progenitors. *Eur J Pharmacol* 338: 277-87.

Longuemare MC, Swanson RA (1997) Net glutamate release from astrocytes is not induced by extracellular potassium concentrations attainable in brain. *J Neurochem* 69: 879-82.

LoPachin RM, Gaughan CL, Lehning EJ, Kaneko Y, Kelly TM, Blight A (1999) Experimental spinal cord injury: spatiotemporal characterization of elemental concentrations and water contents in axons and neuroglia. *J Neurophysiol* 82: 2143-53.

LoPachin RM, Stys PK (1995) Elemental composition and water content of rat optic nerve myelinated axons and glial cells: effects of in vitro anoxia and reoxygenation. *J Neurosci* 15: 6735-6746.

Matute C (1998) Characteristics of acute and chronic kainate excitotoxic damage to the optic nerve. *Proc Natl Acad Sci U S A* 95: 10229-34.

Matute C, Miledi R (1993) Neurotransmitter receptors and voltage-dependent Ca^{2+} channels encoded by mRNA from the adult corpus callosum. *Proc Natl Acad Sci U S A* 90: 3270-4.

Matute C, Sanchez-Gomez MV, Martinez-Millan L, Miledi R (1997) Glutamate receptor-mediated toxicity in optic nerve oligodendrocytes. *Proc Natl Acad Sci U S A* 94: 8830-8835.

McDonald JW, Althomsons SP, Hyrc KL, Choi DW, Goldberg MP (1998) Oligodendrocytes from forebrain are highly vulnerable to AMPA/kainate receptor-mediated excitotoxicity. *Nat Med* 4: 291-297.

McMahon HT, Nicholls DG (1991) Transmitter glutamate release from isolated nerve terminals: evidence for biphasic release and triggering by localized Ca^{2+} . *J Neurochem* 56: 86-94.

Nicotera P, Bellomo G, Orrenius S (1992) Calcium-mediated mechanisms in chemically induced cell death. *Annu. Rev Pharmacol Toxicol* 32: 449-70.

Obrenovitch TP (1996) Origins of glutamate release in ischaemia. *Acta Neurochir (Suppl)* 66: 50-5.

Obrenovitch TP, Urenjak J (1997) Is high extracellular glutamate the key to excitotoxicity in traumatic brain injury? *J Neurotrauma* 14: 677-98.

Omar AI, Senatorov VV, Hu B (2000) Ethidium bromide staining reveals rapid cell dispersion in the rat dentate gyrus following ouabain-induced injury. *Neuroscience* 95: 73-80.

Paternain AV, Morales M, Lerma J (1995) Selective antagonism of AMPA receptors unmasks kainate receptor-mediated responses in hippocampal neurons. *Neuron* 14: 185-189.

Peghini P, Janzen J, Stoffel W (1997) Glutamate transporter EAAC-1-deficient mice develop dicarboxylic aminoaciduria and behavioral abnormalities but no neurodegeneration. *Embo J* 16: 3822-32.

Pitt D, Werner P, Raine CS (2000) Glutamate excitotoxicity in a model of multiple sclerosis. *Nat Med* 6: 67-70.

Roettger V, Lipton P (1996) Mechanism of glutamate release from rat hippocampal slices during in vitro ischemia. *Neuroscience* 75: 677-685.

Rosenberg LJ, Teng YD, Wrathall JR (1999) 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f)quinoxaline reduces glial loss and acute white matter pathology after experimental spinal cord contusion. *J Neurosci* 19: 464-475.

Rossi DJ, Oshima T, Attwell D (2000) Glutamate release in severe brain ischaemia is mainly by reversed uptake. *Nature* 403: 316-321.

Rothstein JD, Dykes-Hoberg M, Pardo CA, Bristol LA, Jin L, Kuncl RW, Kanai Y, Hediger MA, Wang Y, Schielke JP, Welty DF (1996) Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate. *Neuron* 16: 675-86.

Rutledge EM, Kimelberg HK (1996) Release of [3H]-D-aspartate from primary astrocyte cultures in response to raised external potassium. *J Neurosci* 16: 7803-11.

Sanchez-Prieto J, Gonzalez P (1988) Occurrence of a large Ca^{2+} -independent release of glutamate during anoxia in isolated nerve terminals (synaptosomes). *J Neurochem* 50: 1322-4.

Santos MS, Moreno AJ, Carvalho AP (1996) Relationships between ATP depletion, membrane potential, and the release of neurotransmitters in rat nerve terminals. An in vitro study under conditions that mimic anoxia, hypoglycemia, and ischemia. *Stroke* 27: 941-50.

Siesjö BK (1986) Calcium and ischemic brain damage. *Eur Neurol* 25: 45-56.

Steinhauser C, Gallo V (1996) News on glutamate receptors in glial cells. *Trends Neurosci* 19: 339-45.

Stier C, Skorka G, Sohr R, Ott T (1996) Time course and role of extracellular Ca^{2+} in veratridine-induced glutamate release. *Neuroreport* 7: 401-4.

Stys PK (1994) WaveTrak: a data acquisition system and waveform database for the Macintosh. *Sci. Comput. Automation* 10: 19-24.

Stys PK (1998) Anoxic and ischemic injury of myelinated axons in CNS white matter: from mechanistic concepts to therapeutics. *J Cereb Blood Flow Metab* 18: 2-25.

Stys PK, Waxman SG, Ransom BR (1992) Ionic mechanisms of anoxic injury in mammalian CNS white matter: role of Na⁺ channels and Na⁺-Ca²⁺ exchanger. *J Neurosci* 12: 430-439.

Sutherland ML, Delaney TA, Noebels JL (1996) Glutamate transporter mRNA expression in proliferative zones of the developing and adult murine CNS. *J Neurosci* 16: 2191-2207.

Szatkowski M, Attwell D (1994) Triggering and execution of neuronal death in brain ischaemia: two phases of glutamate release by different mechanisms. *Trends Neurosci* 17: 359-65.

Szatkowski M, Barbour B, Attwell D (1990) Non-vesicular release of glutamate from glial cells by reversed electrogenic glutamate uptake. *Nature* 348: 443-6.

Tachibana M, Okada T (1991) Release of endogenous excitatory amino acids from ON-type bipolar cells isolated from the goldfish retina. *J Neurosci* 11: 2199-208.

Takahashi M, Billups B, Rossi D, Sarantis M, Hamann M, Attwell D (1997) The role of glutamate transporters in glutamate homeostasis in the brain. *J Exp Biol* 200: 401-9.

Tanaka K, Watase K, Manabe T, Yamada K, Watanabe M, Takahashi K, Iwama H, Nishikawa T, Ichihara N, Kikuchi T, Okuyama S, Kawashima N, Hori S, Takimoto M, Wada K (1997) Epilepsy and exacerbation of brain injury in mice lacking the glutamate transporter GLT-1. *Science* 276: 1699-702.

Taylor CP, Burke SP, Weber ML (1995) Hippocampal slices: glutamate overflow and cellular damage from ischemia are reduced by sodium-channel blockade. *J Neurosci Methods* 59: 121-8.

Teng YD, Wrathall JR (1997) Local blockade of sodium channels by tetrodotoxin ameliorates tissue loss and long-term functional deficits resulting from experimental spinal cord injury. *J Neurosci* 17: 4359-4366.

Trapp BD, Bernier L, Andrews SB, Colman DR (1988) Cellular and subcellular distribution of 2',3'-cyclic nucleotide 3'-phosphodiesterase and its mRNA in the rat central nervous system. *J Neurochem* 51: 859-868.

Tymianski M, Tator CH (1996) Normal and abnormal calcium homeostasis in neurons: a basis for the pathophysiology of traumatic and ischemic central nervous system injury. *Neurosurgery* 38: 1176-95.

Vandenberg RJ (1998) Molecular pharmacology and physiology of glutamate transporters in the central nervous system. *Clin Exp Pharmacol Physiol* 25: 393-400.

Waxman SG (1992) Demyelination in spinal cord injury and multiple sclerosis: what can we do to enhance functional recovery? *J Neurotrauma* 9 (Suppl 1): S105-17.

Westerink BH, Damsma G, de Vries JB (1989) Effect of ouabain applied by intrastriatal microdialysis on the in vivo release of dopamine, acetylcholine, and amino acids in the brain of conscious rats. *J Neurochem* 52: 705-12.

Wrathall JR, Choiniere D, Teng YD (1994) Dose-dependent reduction of tissue loss and functional impairment after spinal cord trauma with the AMPA/kainate antagonist NBQX. *J Neurosci* 14: 6598-6607.

Yoshioka A, Bacskai B, Pleasure D (1996) Pathophysiology of oligodendroglial excitotoxicity. *J Neurosci Res* 46: 427-37.

Yoshioka A, Hardy M, Younkin DP, Grinspan JB, Stern JL, Pleasure D (1995) Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors mediate excitotoxicity in the oligodendroglial lineage. *J Neurochem* 64: 2442-8.

Zerangue N, Kavanaugh MP (1996) Flux coupling in a neuronal glutamate transporter. *Nature* 383: 634-637.

Figure 1 Bar graphs representing mean peak amplitudes and tracings illustrating compound action potentials recorded from *in vitro* dorsal column slices in normal control or following ouabain plus high K^+ perfusion with or without Ca^{2+} . Peak amplitudes were normalized to baseline responses recorded before drug application (panel A). Ouabain (500 μM) was applied for 30 min, followed by 60 min of the same concentration of this drug + high K^+ (129 mM, equimolar replacement with Na^+). Then the slices were washed with aCSF for 2 hrs. To examine the role of external Ca^{2+} on ion collapse-induced injury, zero- Ca^{2+} perfusate (replaced by equimolar Mg^{2+}) was applied 30 min prior to ouabain and maintained until 30 min after ouabain + high K^+ perfusion. In the time-matched normal control (white bars), the amplitude remained stable throughout the experiments. In the slices exposed to ouabain + high K^+ , amplitudes recovered to $\approx 43\%$ of pre-drug control following 2 hr aCSF wash (*gray bars*), whereas recovery was significantly enhanced to $\approx 87\%$ of control by removing Ca^{2+} from the perfusate (black bars). Panels *B* shows representative compound action potential tracings obtained following aCSF wash. ** $p < 0.01$ compared to time-matched readings of slices injured in presence of external Ca^{2+} .

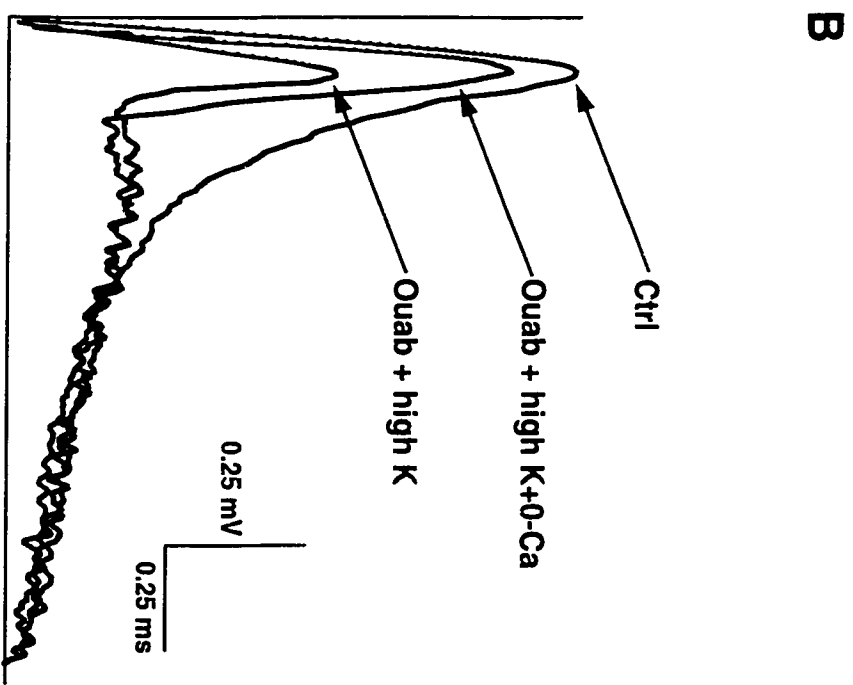
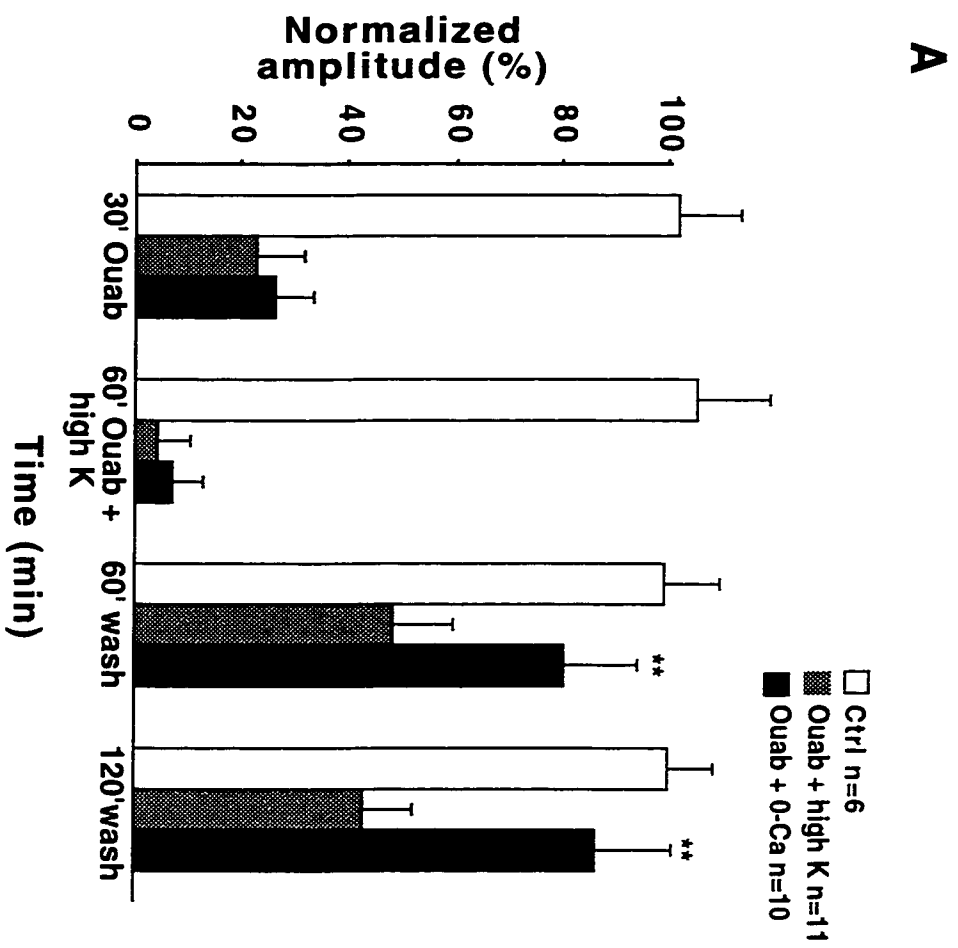


Figure 1

Figure 2 Bar graphs illustrating the effects of ionotropic glutamate receptor antagonists on the recovery of compound action potentials following inhibition of Na⁺-K⁺-ATPase and high K⁺ application. The antagonists were applied beginning 30 min before and continued until 30 min after ouabain plus high K⁺ administration. Both kynurenic acid (KYN, 1 mM) and GYKI52466 (GYKI, 30 μM) significantly improved compound action potential amplitudes following ouabain (500 μM) and high K⁺ treatment (Panel A). Panels *B* shows representative compound action potential tracings after aCSF wash from each group. * $p < 0.05$, ** $p < 0.01$ compared to time-matched readings of slices injured in absence of glutamate receptor antagonists.

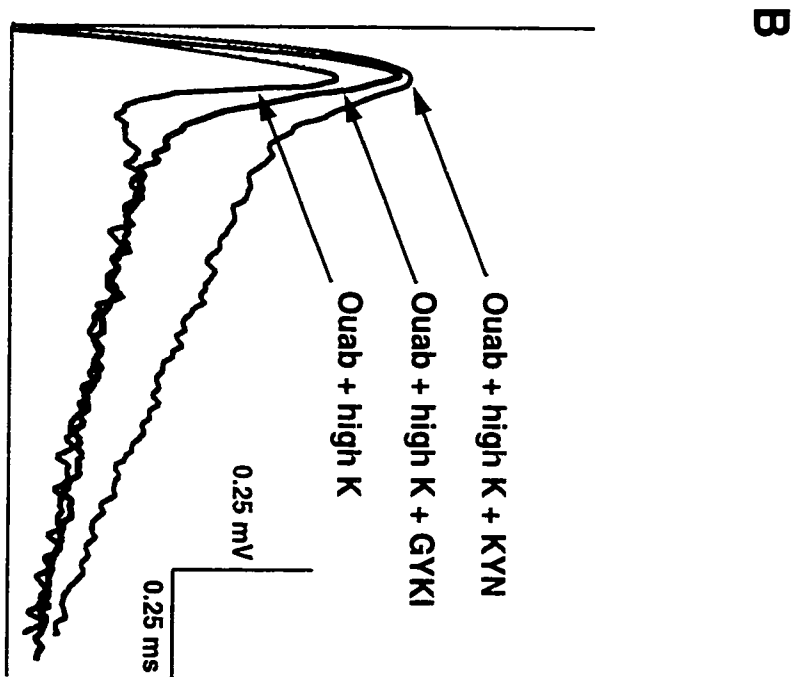
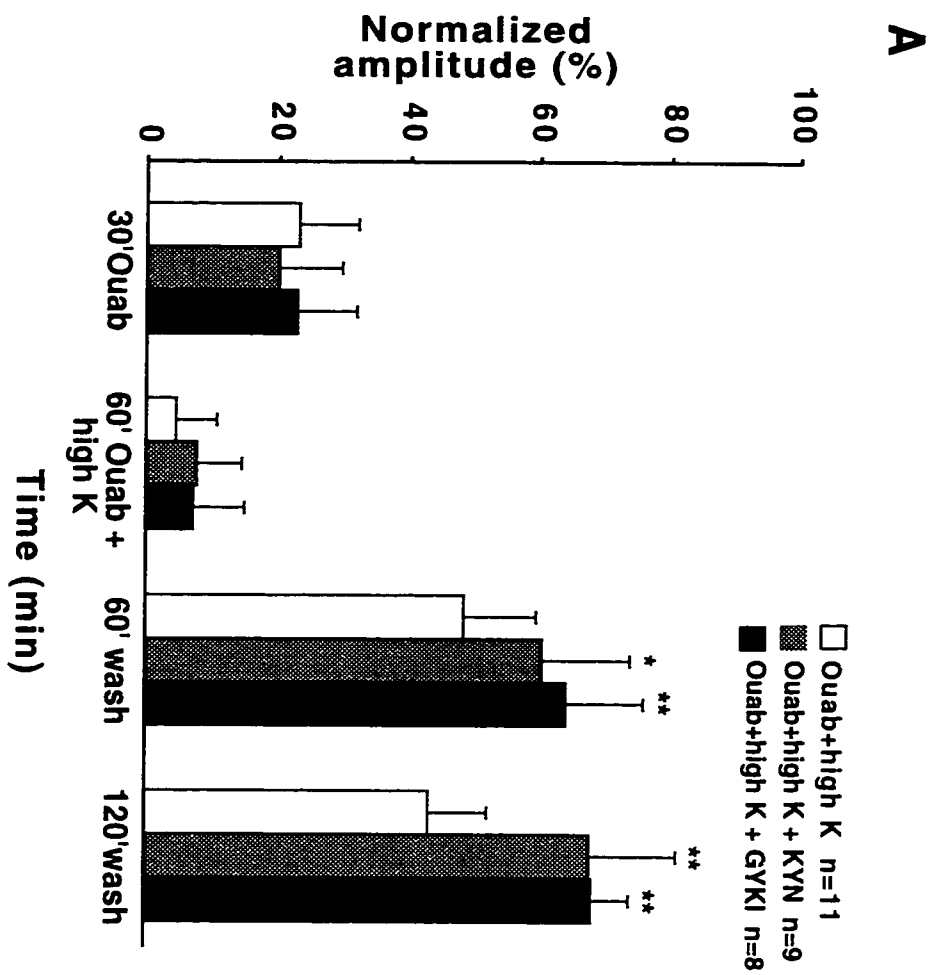


Figure 2

Figure 3 Role of glutamate transport or Na⁺-Ca²⁺ exchanger inhibitors in the recovery of compound action potentials following ouabain and high K⁺ application. The glutamate transport inhibitor was applied beginning 30 min before and continued until 30 min after ouabain plus high K⁺ application. L-trans-pyrrolidine-2,4-dicarboxylic acid (1 mM, PDC) significantly improved compound action potential amplitudes following ouabain (500 μM) and high K⁺ perfusion (panel A). Panels B shows representative compound action potential tracings following aCSF wash. Inhibition of Na⁺-Ca²⁺ exchanger with KB-R7943 (10 μM) was not protective, AMPA receptor blockade with GYKI32466 (30 μM) significantly improved CAP amplitudes (panel C). * p< 0.05, ** p<0.01 compared to time-matched readings of slices without glutamate transport inhibitors.

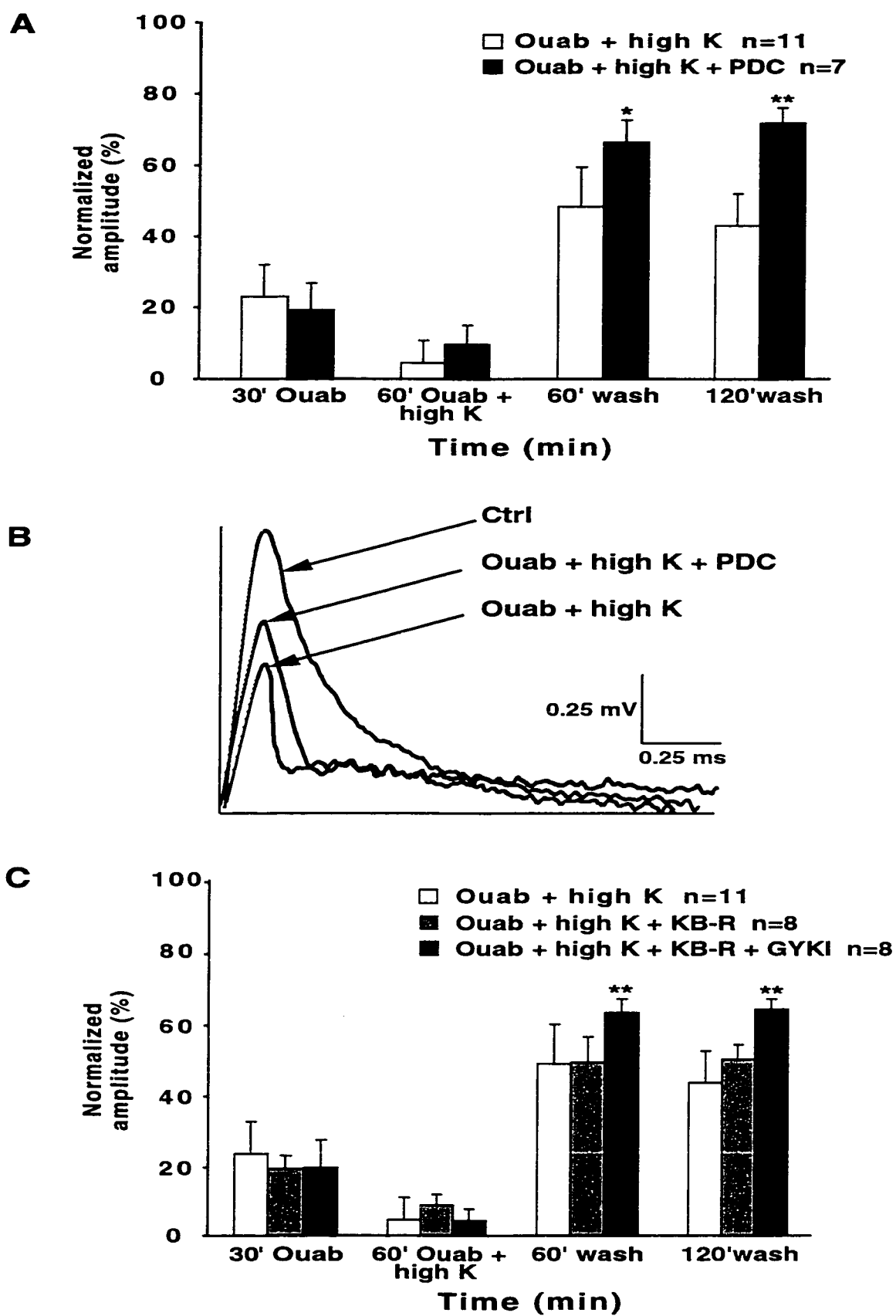


Figure 3

Figure 4 Confocal imaging and bar graph to semi-quantitatively estimate glutamate signal in dorsal column white matter slices. Immunohistochemistry of dorsal column white matter doubly stained for standard markers (neurofilament, CNPase and GFAP) and glutamate in control (panel A, D and G), ouabain + high K⁺ (panel B, E and H) and glutamate transport inhibitor-treated (panel C, F and I) slices. The markers for neurofilament, CNPase and GFAP illustrate axon cylinders (panel A, B and C), oligodendroglial (panel D, E and F) and astrocytic (panel G, H and I) somata and processes, respectively. Regions of interest outlined by each of the standard markers were analyzed for fluorescence intensity from the glutamate channel (red), thus producing a semi-quantitative estimate of glutamate in axon cylinders, oligodendrocytes and astrocytes. Mean glutamate fluorescence from each of these three white matter elements is plotted in the bar graphs (panel J). Glutamate was significantly reduced in axon cylinders (gray in left bar graph), and oligodendrocytes (gray in middle bar graph), but not in astrocytes (gray in right bar graph) following exposure to ouabain (500 μ M) and high K⁺ (129 mM, equimolar replacement) compared with time-matched slices incubated in aCSF without any drugs. However, treatment with glutamate transport inhibitor L-trans-pyrrolidine-2,4-dicarboxylic acid (1 mM, PDC) largely prevented the decrease of glutamate signal from axon cylinder (black in left bar graph). The number of “n” represents sections of imaging analyzed from each of cell components in each group. * p<0.01. Scale bars 10 μ m.

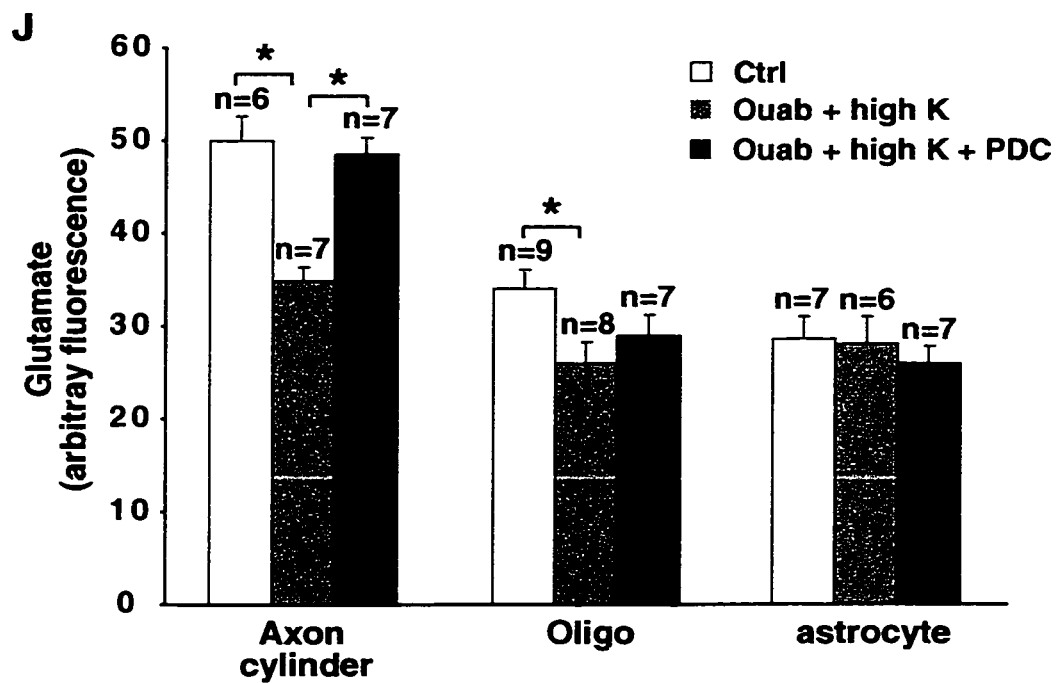
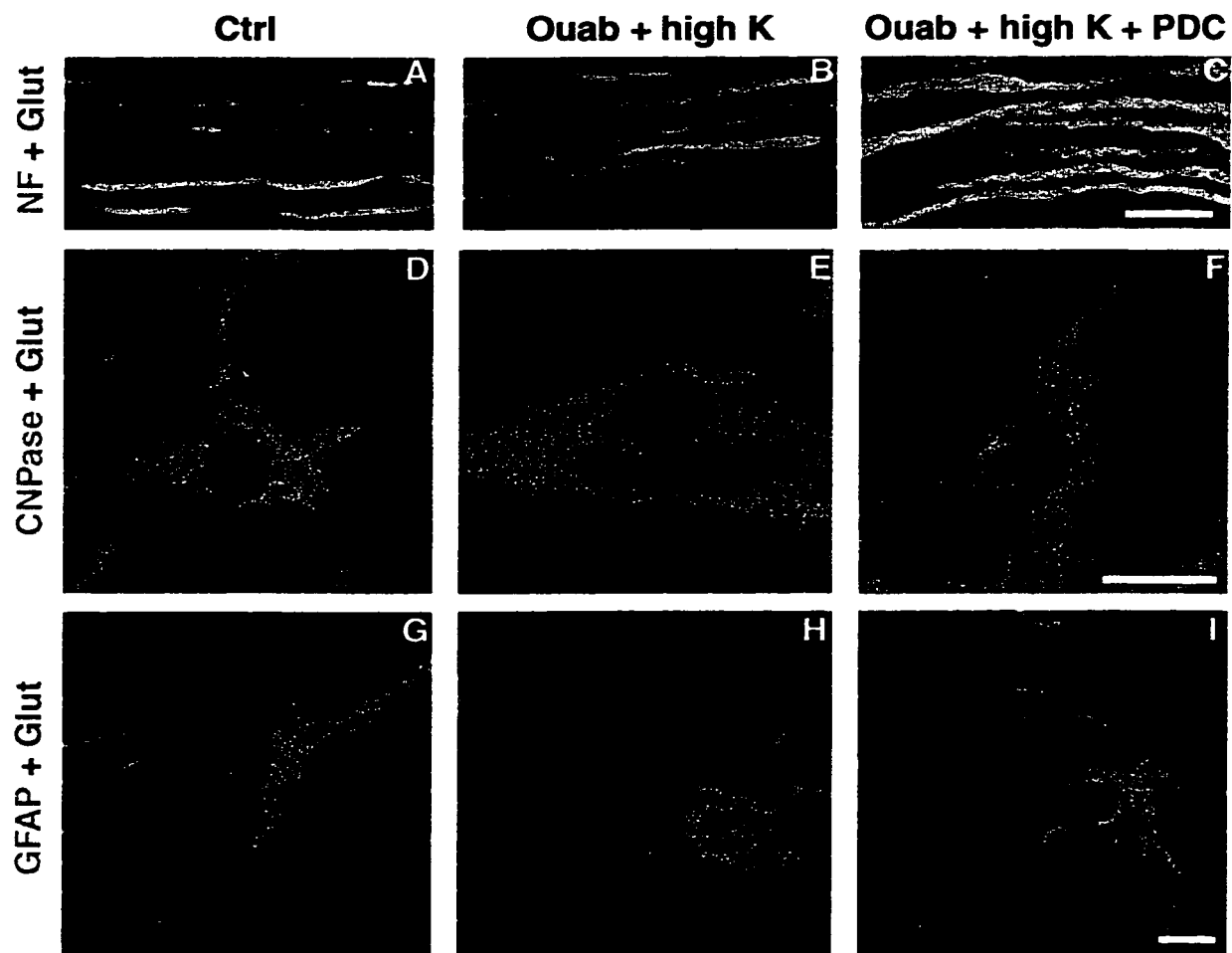


Figure 4

Chapter 4

Novel injury mechanism in anoxia and trauma of spinal cord white matter: glutamate release via reverse Na⁺-dependent glutamate transport

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ABSTRACT

Spinal cord injury is a devastating condition, with much of the clinical disability resulting from disruption of white matter tracts. Recent reports suggest a component of glutamate excitotoxicity in spinal cord injury. In this study, the role of glutamate and mechanism of release of this excitotoxin were investigated in rat dorsal column slices subjected to 60 min of anoxia or 15 s of mechanical compression at a force of 2 g *in vitro*. The broad spectrum glutamate antagonist kynurenic acid (1 mM) or the selective AMPA antagonist GYKI52466 (30 μ M) were protective against anoxia (compound action potential amplitude recovered to 56% vs. 27% without drug). GYKI52466 was also effective against trauma (65% vs. 35%). Inhibition of Na⁺-dependent glutamate transport with dihydrokainate or L-trans-pyrrolidine-2,4-dicarboxylic acid (1 mM each) protected against anoxia (65-75% vs. 25%) and trauma (70% vs. 35%). The depletion of cytosolic glutamate in oligodendrocytes and axon cylinders by anoxia was completely prevented by glutamate transport inhibition. Immunohistochemistry revealed that a large component of injury occurred in the myelin sheath, and was prevented by AMPA receptor blockade or glutamate transport inhibitors. We conclude that release of glutamate by reversal of Na⁺-dependent glutamate transport with subsequent activation of AMPA receptors is an important mechanism in spinal cord white matter anoxic and traumatic injury.

Keywords

Spinal cord injury, axon, anoxia, trauma, AMPA, Na⁺-glutamate transport, myelin, dihydrokainate, L-trans-pyrrolidine-2,4-dicarboxylic acid, GYKI52466, kynurenic acid

INTRODUCTION

White matter tracts within the mammalian central nervous system (CNS) play the very important role of transmitting information to and from neurons in the CNS. The spinal cord, arguably the most important white matter tract, is subject to traumatic injury with more than 10,000 new cases occurring in the U.S. alone (Gibson, 1992). While both the central gray matter in the cord as well as surrounding axonal tracts suffer damage from the mechanical trauma and secondary ischemia (Tator and Koyanagi, 1997), disruption of axonal connections spanning even a small segment can result in severe and widespread disability. The underlying mechanisms leading to axonal dysfunction in spinal cord injury (SCI) are poorly understood and current treatment is of limited efficacy. Therefore understanding how axons are irreversibly damaged in this condition is of paramount importance in order to devise more effective treatments in the acute phase.

Recent reports using *in vitro* and *in vivo* SCI models indicate that voltage-gated Na⁺ channels play an important role in mediating cellular injury in SCI (Agrawal and Fehlings, 1996; Teng and Wrathall, 1997), similar to observations in anoxic axons (Imaizumi et al., 1997; Stys et al., 1992). In addition, injury is also dependent on excitotoxic mechanisms involving AMPA/kainate receptors (Agrawal and Fehlings, 1997; Wrathall et al., 1997). However, the precise cellular targets for glutamate toxicity in white matter are not known, nor is there an explanation of how glutamate might be released in this tissue devoid of synaptic elements. In this study, we demonstrate that in isolated spinal dorsal columns endogenous glutamate is released by reversal of Na⁺-dependent glutamate transport. We also show that the myelin sheath is a target for

glutamate-mediated excitotoxicity through activation of AMPA receptors. This mechanism would unite and explain the observations that both Na⁺ channel blockade or AMPA antagonists are neuroprotective in SCI, as Na⁺ entry through the former route would induce reverse glutamate transport and cause release of potentially large amounts of this excitotoxin from cytosolic compartments.

MATERIALS AND METHODS

Electrophysiology. Adult Long-Evans male rats (200-250 gm) were anesthetized with sodium pentobarbital, and a laminectomy was performed between T3 and T11. Rats were then perfused intra-aortically with 500 ml of choline-substituted zero-Na⁺, zero-Ca²⁺ solution. A 30 mm section of spinal cord was rapidly removed and placed in cold (4-6 °C) zero-Na⁺, zero-Ca²⁺ solution bubbled with 95% O₂ and 5% CO₂. Dorsal column sections were excised and placed in an interface recording chamber bathed in Ca²⁺-free artificial CSF (aCSF) and slowly warmed to 37°C. Perfusate was then switched to aCSF (in mM: 126 NaCl, 3.0 KCl, 2.0 MgSO₄, 26 NaHCO₃, 1.25 NaH₂PO₄, 2.0 CaCl₂, and 10 dextrose, pH 7.4), and control readings were taken 30 min later.

Propagated compound action potentials (CAPs) were evoked using a bipolar silver wire stimulating electrode (50 µsec and typically 70 V) delivered once every 30 min, and extracellular recordings were performed using large-tipped glass microelectrodes filled with 150 mM NaCl (Fig. 1). To allow recording of multiple slices during a single experiment, the stimulation and recording sites were marked with a small amount of neutral red dye to allow accurate repositioning of the electrodes. Evoked CAPs were

digitized, stored, and analyzed using WaveTrak software without rectification (Stys, 1994). The functional integrity of the dorsal column was quantitated by measuring peak CAP amplitude.

Potential direct effects of glutamate transport inhibitors on AMPA currents were studied by patch clamp in cultured rat cortical neurons as previously described (Mealing et al., 1999). The bathing solution contained (in mM): 140 NaCl, 5 KCl, 1 CaCl₂, 10 HEPES, 3 glucose, and 0.001 TTX, 0.001 strychnine, pH 7.4. The pipette solution contained (in mM): 140 CsCl, 1.1 EGTA, 10 HEPES, and 2 Mg-ATP, pH 7.2. Solutions were applied to the cell through a computer-controlled manifold. Whole-cell currents were measured at a holding potential of 60 mV after a 0.5 sec application of cyclothiazide (100 μ M), then 1 sec AMPA (100 μ M) plus cyclothiazide, followed 10 sec later by a second application of AMPA plus cyclothiazide with or without 1 mM dihydrokainic acid or L-*trans*-pyrrolidine-2,4-dicarboxylic acid (Tocris Cookson, Bristol, UK).

In vitro anoxia and SCI. Drug-containing solutions were applied beginning 60 min before injury, and continued until 15 min post-injury after which tissue was washed with aCSF. Injury was induced by anoxia or trauma. Anoxia was achieved by switching to a 95% N₂ / 5%CO₂ atmosphere for 60 min, then reoxygenated for 2 hrs. Trauma was induced by compression with a custom-made aneurysm clip calibrated to a closing force of 2 g (David Walsh, Oakville, Ont.), applied for 15 s between the stimulation and recording sites (Agrawal and Fehlings, 1997). GYKI52466 (RBI), dihydrokainic acid and L-*trans*-pyrrolidine-2,4-dicarboxylic were dissolved in 0.1N HCl (GYKI52466) or

0.1N NaOH, then added to aCSF to the desired final concentration. Kynurenic acid (RBI) was dissolved directly into aCSF.

Immunohistochemistry of glutamate and damaged myelin. To directly examine to what extent the myelin sheath was affected by our injury paradigms, we used rabbit antiserum raised against degenerated myelin basic protein (anti-EP; a generous gift from Dr. Pat McGeer, University of British Columbia), which was found to stain damaged, but not intact, white matter regions (Matsuo et al., 1997). Tissue was fixed in 4% paraformaldehyde for 24 hr and then cryoprotected for 48 hr in PBS, pH 7.4, containing 20% glycerol at 4°C. Slices were then dissected into smaller pieces and preincubated in 10% Triton X-100 for 30 min, followed by 4% normal goat serum (NGS) with 0.1% Triton X-100, and PBS for blocking for 1 hr at room temperature. The sections were incubated for 24 hr at 4°C with primary antiserum diluted in 2% NGS with 0.1% Triton X-100 and PBS at a concentration of 1:100 for anti-EP and for anti-mouse neurofilament 160. Alexa 594 goat anti-rabbit (1:200) and Alexa 488 goat anti-mouse (1:400; Molecular Probes, Eugene, OR) were used for secondaries. Controls consisted of primary or secondary antibodies omitted.

The protocol for glutamate immunohistochemistry was similar, except that 0.5% glutaraldehyde was used as an additional fixative. A rabbit anti-glutamate polyclonal antibody (Chemicon, Temecula, CA) was used at 1:500 dilution to label cytosolic glutamate. Double staining with monoclonals against neurofilament 160 (Sigma, St. Louis, MO), 2'3'-cyclic nucleotide 3'-phosphohydrolase (CNPase, Chemicon), and anti-

glial fibrillary acidic protein (GFAP; Boehringer Mannheim, Indianapolis, IN) allowed localization of axon cylinders, oligodendrocytes (Trapp et al., 1988), and astrocytes, respectively. Antiserum concentrations, incubation times, and all tissue preparation were identical between groups to reduce artifactual changes in observed fluorescence. In addition, confocal parameters (pinhole size, laser power, gain, and black level) were constant to allow for valid comparisons between treatment groups. Images were analyzed blindly using NIH Image 1.61 (<http://rsb.info.nih.gov/nih-image/default.html>). Regions of interest were selected according to reference labels (i.e., neurofilament 160, CNPase, and GFAP) and mean fluorescence values, reflecting glutamate concentration in that area, computed from the "glutamate" channel.

Immunohistochemistry of glutamate transporters. Rats were perfused intra-aortically with cold 0.1 M PBS, pH 7.4, followed by 4% paraformaldehyde in PBS after laminectomy under pentobarbital anesthesia. The process was similar to the previous section, except that the slices were also pretreated with 95% ethanol and 5% acetic acid for 60 min. The primary antibodies were diluted in 2% NGS with 0.1% Triton X-100 and PBS at a concentration of 5 µg/ml for anti-GLT1 (N terminus; Alpha Diagnostic International, San Antonio, TX), 0.98 µg/ml for anti-GLAST (N terminus), 0.49 µg/ml for anti-EAAC1 (C terminus; courtesy of Dr. Jeffrey Rothstein, Johns Hopkins University, Baltimore, MD) (Rothstein et al., 1994; Furuta et al., 1997), 1:2000 for anti-myelin basic protein (MBP; Sternberger Monoclonals, Lutherville, MD), and 1:100 for GFAP. Images were collected on a Bio-Rad (Hercules, CA) 1024 confocal laser scanning microscope with a 60x oil-immersion lens (Olympus Optical, Tokyo, Japan).

Statistics. All data are expressed as means $\pm$ standard deviation. Statistical differences were calculated by ANOVA with Dunnett's test for comparisons with a common control group, or ANOVA with Bonferroni correction for multiple comparisons. Reported n values represent number of individual dorsal column slices studied with each treatment.

RESULTS

AMPA receptors contribute to injury of dorsal white matter

Electrophysiological recording of dorsal column slices showed a reduction of CAP amplitude to ~25% of control after 60 min of anoxia followed by reoxygenation and to ~35% of control after a 15 sec traumatic clip compression (Fig. 2). Uninjured controls displayed <5% change in mean CAP amplitude during 3 hr *in vitro* (Fig. 1). To confirm a role of glutamate receptors, tissue was exposed to 1mM kynurenic acid, a broad-spectrum inhibitor of both NMDA and non-NMDA ionotropic receptors. This agent improved recovery of CAP amplitude after 60 min of anoxia to 56% of control versus 27% without drug ($p < 0.01$; Fig. 2). GYKI52466 (30 μ M), a selective AMPA glutamate receptor antagonist (Paternain et al., 1995), significantly improved the recovery of CAP amplitude after anoxia (56 vs 27% of control CAP amplitude; $p < 0.01$) or trauma (65 vs 35% without drug; $p < 0.01$), indicating that glutamate partially contributes to white matter injury through AMPA receptors during anoxia or trauma.

Figure 3 shows representative fluorescence images of normal dorsal column slices incubated under normoxic conditions for 3 hr *in vitro* (Fig. 3A) and tissue injured by

anoxia (Fig. 3B-E) or SCI (Fig. 3F,G). *Green* signal is neurofilament outlining axon cylinders, and the *red* channel indicates damaged myelin stained with serum specific for degenerated myelin basic protein (see Materials and Methods). Figure 3, D and E, shows that myelin damage was largely prevented by AMPA receptor blockade with GYKI52466.

Reverse Na⁺-dependent glutamate transport contributes to glutamate release during anoxia and SCI

The results presented above suggest that endogenous glutamate is released from cytoplasmic compartments in isolated spinal white matter. Anoxia or trauma causes disturbances of ionic gradients and membrane depolarization in white matter tracts (LoPachin and Stys, 1995; Leppanen and Stys, 1997; Blight and LoPachin, 1998) that may induce release of glutamate in a Ca²⁺-independent manner through reversal of Na⁺-dependent glutamate transport, as has been shown in gray matter (Roettger and Lipton, 1996). To test this hypothesis, we examined the effect of glutamate transport inhibition on the recovery of dorsal white matter after *in vitro* anoxia or SCI. L-*trans*-Pyrrolidine-2,4-dicarboxylic acid is a transportable antagonist (Griffiths et al., 1994); therefore the tissue was preloaded, probably by heteroexchange with glutamate, so that sufficient levels of inhibitor would be available at the cytoplasmic face to inhibit glutamate release. In contrast, dihydrokainate is a nontransportable inhibitor of the GLT1 subtype of glutamate transporter acting at the extracellular surface (Arriza et al., 1994). Both inhibitors (applied at 1 mM) were significantly neuroprotective against *in vitro* anoxia or SCI, increasing recovery of CAP amplitudes twofold to threefold compared with

untreated injured tissue (Fig. 4A,B). The degree of myelin damage after *in vitro* SCI was markedly reduced by glutamate transport inhibition (Fig. 3F,G), although the disruption of axon cylinders shown by neurofilament staining appeared unchanged. Confocal fluorescence was used to estimate semiquantitatively the cytosolic glutamate concentrations in axon cylinders and glial cell bodies and processes after 1 hr of anoxia. Figure 4E shows that glutamate levels were significantly reduced in axon cylinders and oligodendrocytes by anoxia, and this reduction was completely reversed by L-*trans*-pyrrolidine-2,4-dicarboxylic acid. Astrocytic glutamate was not significantly altered by anoxia.

To exclude the possibility that glutamate transport inhibitors exerted their neuroprotective actions through interaction directly with AMPA receptors, the effects of these agents on AMPA-induced currents were measured by patch clamp in cultured neurons. Steady-state currents were increased slightly to 111 ± 5 and $120 \pm 11\%$ of control by 1 mM L-*trans*-pyrrolidine-2,4-dicarboxylic acid and dihydrokainate, respectively (data not shown). This was not statistically significant.

Dorsal column white matter possesses three subtypes of Na⁺-dependent glutamate transporters: GLT1, GLAST and EAAC1

The previous results provide pharmacological evidence for the presence of Na⁺-dependent glutamate transporters in spinal white matter. More direct evidence was provided by immunohistochemistry using specific antisera raised against the GLT1, GLAST, and EAAC1 subtypes. Figure 5 illustrates the distributions of the various

isoforms. Consistent with previous reports (Rothstein et al., 1994), GLT1 was found in GFAP-positive astrocytes at high density (Fig. 5B), with fainter stain seen within the axoplasm of myelinated axons (Fig. 5A). GLT1 was not found in myelin, in contrast to GLAST, which was present throughout the thickness of the sheath (Fig. 5C). GLAST was also observed in astrocytes (Fig. 5D). EAAC1, a predominantly neuronal isoform, was not present in myelin, and label was only weakly observed in some GFAP-positive astrocytes (Fig. 5F). There was considerable intervening stain outside myelin and astrocytic regions (Fig. 5E), potentially associated with unmyelinated axons, axoplasm of myelinated fibers, or oligodendroglial processes. The precise localization of this signal was not investigated further.

DISCUSSION

Central myelinated axons are susceptible to a variety of insults, the commonest being anoxia/ischemia, trauma and demyelination. Indeed, these seemingly disparate injury modalities may share common mechanisms; for example, traumatic spinal cord injury consists of both the acute mechanical disruption of spinal axons which is followed by a delayed ischemic component (Tator and Koyanagi, 1997). The cellular mechanisms of axonal injury are not as well understood as those in gray matter, where excitotoxicity leading to Ca^{2+} -mediated injury, free radical generation and delayed apoptosis are thought to be the main avenues by which neurons succumb to anoxia/ischemia. In anoxic central myelinated axons, in contrast, excessive Na^+ influx through non-inactivating Na^+ channels causes Ca^{2+} overload largely through reverse Na^+ - Ca^{2+} exchange. The excessive Ca^{2+} influx in turn triggers a variety of Ca^{2+} -dependent biochemical pathways leading to

irreversible axonal damage (Imaizumi et al., 1997; Stys and LoPachin, 1998).

The precise role of glutamate-mediated excitotoxicity in white matter injury is poorly understood. The NMDA receptor antagonist ketamine failed to show any neuroprotective effects against *in vitro* optic nerve anoxia at concentrations low enough to ensure relative specificity for these receptors (Ransom et al., 1990). Recent reports, however, indicate that glutamate, acting through non-NMDA receptors, may play a direct role in white matter injury. For example, white matter oligodendroglia possess both AMPA and kainate receptors at densities sufficient to cause significant injury when activated by specific agonists (Matute et al., 1997; McDonald et al., 1998). In addition, *in vitro* oxygen/glucose deprivation causes damage to cultured oligodendroglia that is dependent on AMPA/kainate receptors (McDonald et al., 1998). By extension, it may be possible that the myelin sheath itself possesses AMPA/kainate receptors and may be directly susceptible to injury from high concentrations of ambient glutamate released from compromised glia or axon cylinders.

Traumatic injury of spinal cord white matter also appears to depend on glutamate. Using an *in vivo* contusive model, Wrathall and colleagues found a reduction in white matter pathology (Rosenberg et al., 1999), and a parallel behavioral improvement (Wrathall et al., 1994), in animals treated with the AMPA/kainate antagonist NBQX. The neuroprotective effect of AMPA/kainate receptor blockade was also observed in an *in vitro* model of isolated dorsal column compression. Without the potentially confounding influence of adjacent gray matter, dorsal columns were found to be injured by

exogenously applied AMPA or kainate, and compressive injury was dependent in part on AMPA/kainate receptor activation as evidenced by a partial neuroprotective effect of CNQX or NBQX (Agrawal and Fehlings, 1997). Taken together, these findings implicate AMPA/kainate receptor activation as one component of the injury cascade in white matter. In this study, we wished to explore both the mode of glutamate release, target receptors and loci of injury in spinal white matter anoxia and trauma.

Using an electrophysiological measure of functional integrity, a one hour anoxic exposure followed by one or two hours of reoxygenation resulted in irreversible depression of CAP amplitude to 27% of pre-anoxic control values. This is very close to the level reported in a similar *in vitro* model (Imaizumi et al., 1997). The broad spectrum glutamate receptor antagonist kynurenic acid or the relatively specific AMPA receptor antagonist GYKI52466 (Paternain et al., 1995), both provided significant and virtually identical neuroprotection from anoxia, with CAP amplitudes recovering to over twice that seen without antagonist. This is consistent with a role of AMPA, but not NMDA, receptors in this paradigm. AMPA receptors also appeared to play a significant role in trauma. A 2 g compression injury *in vitro* resulted in an irreversible reduction of CAP amplitude to 35% of control levels in our study. This injury was identical to that found in a recent *in vitro* study using guinea pig spinal cord slices at physiological temperature (Blight and LoPachin, 1998), but was far greater than observations of Agrawal and Fehlings (1997) who found reductions of CAP amplitude to only 70% of control. However the latter group performed their experiments at temperatures significantly below physiological (typically 25 °C), and it is likely that the hypothermia reduced the degree of

injury. Despite a greater degree of damage at physiological temperature in our experiments, GYKI52466 also conferred marked neuroprotection following clip compression. Moreover, in contrast to a previous report (Agrawal and Fehlings, 1997), the protective effect was sustained for the duration of our *in vitro* recording. Together, our data strongly implicate receptors of the AMPA class in both anoxic and traumatic injury of spinal dorsal columns.

Electrophysiology provides a sensitive means to study the function of axonal tracts, but gives little information about the structural integrity of subcellular elements. We used antiserum raised against an amino acid sequence of myelin basic protein that is inaccessible in normal myelin, but is unmasked in degenerated myelin in white matter areas damaged by immune attack or ischemia (Matsuo et al., 1997). Control tissue maintained *in vitro* showed virtually no staining, whereas anoxia or trauma induced obvious myelin damage (Fig. 3B, C and F). These changes were prevented by selective AMPA inhibition indicating not only that this subtype of ionotropic glutamate receptor contributes to myelin injury, but also points to an endogenous source of glutamate. We cannot exclude glutamate-mediated injury to other elements such as astrocytes, oligodendroglial cell bodies and/or the axon cylinder itself. Indeed it is quite possible that glia suffered glutamate-dependent injury as these cells are known to be sensitive to excitotoxic insults mediated by AMPA/kainate receptors (Matute et al., 1997; McDonald et al., 1998).

Because our preparation excludes spinal gray matter, the source of endogenous glutamate must be from glia or axons. Cytoplasm, including axoplasm, is known to contain millimolar concentrations of glutamate that far exceed the low micromolar levels in brain extracellular space (Attwell et al., 1993; Fonnum, 1984). In the absence of synaptic machinery and barring frank membrane rupture, there are two ways that this amino acid could be released: efflux through volume-sensitive anion channels (Rutledge et al., 1998) or by reversal of Na⁺-dependent glutamate transport (Attwell et al., 1993). The latter pathway transports glutamate or aspartate with Na⁺ and H⁺ in exchange for K⁺ in an electrogenic manner (Levy et al., 1998; Zerangue and Kavanaugh, 1996). It follows that a rise in [K⁺]_o and depolarization, along with an increase in [Na⁺]_i, will promote reverse operation of this transporter and the release of glutamate from cytoplasmic compartments. Indeed, central axons damaged by either anoxia or trauma suffer marked depletion of K⁺ and accumulation of Na⁺, with an expected rise in [K⁺]_o and depolarization (Leppanen and Stys, 1997; Ransom et al., 1992), stimuli that would strongly favor reversal of Na⁺-dependent glutamate transport. This hypothesis was supported by the markedly neuroprotective effects of the transport inhibitors dihydrokainate or L-trans-pyrrolidine-2,4-dicarboxylic acid. Moreover, immunohistochemistry for glutamate revealed that anoxic axon cylinders, and to a lesser extent oligodendrocytes, are the main source of endogenous glutamate (Fig. 4E); the efflux of glutamate from these sources, and by inference the rise in [glutamate]_o, is completely prevented by inhibition of Na⁺-dependent glutamate transport. Notably, astrocytic glutamate was unchanged by anoxia, in keeping with the relative resistance of ionic deregulation in this cell type by anoxia alone (Rose et al., 1998).

The above mechanism, possibly representing an exaggeration of a normal physiological release of glutamate as proposed for neonatal optic nerve axons (Kriegler and Chiu, 1993), could also account for the rise in $[\text{glutamate}]_o$ in white matter of ischemic cat brain (Graf et al., 1998), and may contribute to the demyelination and white matter degeneration found after traumatic brain injury (Maxwell et al., 1997; Povlishock and Christman, 1995). Moreover, if myelin is a significant target for glutamate toxicity as our results suggest, the submyelinic spaces where diffusion is restricted could harbor very high glutamate levels. Using anoxic CNS axons as a well characterized example, assuming a depolarization to -30 mV (Leppanen and Stys, 1997), $[\text{Na}^+]_i$, $[\text{Na}^+]_o$, $[\text{K}^+]_i$ and $[\text{K}^+]_o$ of 100, 150, 15 and 15 mM, respectively (LoPachin and Stys, 1995; Ransom et al., 1992), a ratio of $[\text{H}^+]_i$ to $[\text{H}^+]_o$ of 2.5 (with both moving proportionally in the acid direction during injury), $[\text{glutamate}]_i = 3$ mM (Attwell et al., 1993) and a transporter stoichiometry of 3 Na^+ , 1 H^+ , 1 glutamate $^-$: 1 K^+ (Levy et al., 1998), equation 1 predicts that $[\text{glutamate}]_o$ will exceed 230 μM at equilibrium; this ignores any reductions in $[\text{Na}^+]_o$, which would steeply push $[\text{glutamate}]_o$ to even higher levels. Similar ionic deregulation in mechanically injured spinal cord slices (Blight and LoPachin, 1998) implies an equally potent stimulus for reverse Na^+ -glutamate transport in trauma as well.

$$Glu_o = Glu_i \left(\frac{H_i}{H_o} \right)^{\frac{nH}{nGlu}} \left(\frac{Na_i}{Na_o} \right)^{\frac{nNa}{nGlu}} \left(\frac{K_o}{K_i} \right)^{\frac{nK}{nGlu}} e^{\left(\frac{F V_m (nNa + nH - nGlu - nK)}{R T nGlu} \right)} \quad \text{eq.1}$$

where: X_o , X_i are extra- and intracellular ionic concentrations, nX are stoichiometries, V_m is membrane potential.

Pharmacological evidence for the presence of Na^+ -dependent glutamate transport was supported by immunohistochemical staining for all three isoforms in spinal dorsal columns, consistent with previous studies that also found evidence for EAAC1, GLAST and GLT1 in CNS white matter (Choi and Chiu, 1997; Sutherland et al., 1996). Dihydrokainate is a specific inhibitor of the GLT1 isoform (Arriza et al., 1994). The neuroprotective effect of this agent might suggest a purely glial source of glutamate efflux mediated by GLT1; however recent reports indicate that GLT1 may be present in neurons as well (Mennerick et al., 1998; Schmitt et al., 1996). Our data (Fig. 5) also indicate that this isoform is present in the axoplasm, possibly for transport to the terminals or for insertion into the axolemma along the length of the fiber. Demonstration of the presence of glutamate transporters on the axolemma will likely require the high spatial resolution of immunoelectron microscopy.

The present study indicates that the ionic and membrane potential perturbations experienced by dorsal column axons, and possibly glia, in response to *in vitro* anoxia or mechanical trauma, are more than sufficient to induce toxic efflux of glutamate via reversal of Na^+ -dependent glutamate transport. This uncontrolled release of glutamate, potentially into restricted spaces under the myelin, activates AMPA receptors causing damage to the sheath and possibly other structures. This novel mechanism of injury may be very important for the future design of neuroprotectants in SCI, particularly if

molecular design techniques succeed in developing a relatively specific blocker of the glutamate efflux mode mediated by glutamate transporters.

REFERENCES

- Agrawal SK, Fehlings MG (1996) Mechanisms of secondary injury to spinal cord axons in vitro - role of Na^+ , $\text{Na}^+\text{-K}^+\text{-ATPase}$, the $\text{Na}^+\text{-H}^+$ exchanger, and the $\text{Na}^+\text{-Ca}^{2+}$ exchanger. *J Neurosci* 16:545-552.
- Agrawal SK, Fehlings MG (1997) Role of NMDA and non-NMDA ionotropic glutamate receptors in traumatic spinal cord axonal injury. *J Neurosci* 17:1055-1063.
- Arriza JL, Fairman WA, Wadiche JI, Murdoch GH, Kavanaugh MP, Amara SG (1994) Functional comparisons of three glutamate transporter subtypes cloned from human motor cortex. *J Neurosci* 14:5559-69.
- Attwell D, Barbour B, Szatkowski M (1993) Nonvesicular release of neurotransmitter. *Neuron* 11:401-7.
- Blight AR, LoPachin RM (1998) Experimental spinal cord injury: characterization of axonal element and water perturbations. *Soc Neurosci Abstr* 24:251.
- Choi I, Chiu SY (1997) Expression of high-affinity neuronal and glial glutamate transporters in the rat optic nerve. *Glia* 20:184-92.
- Fonnum F (1984) Glutamate: a neurotransmitter in mammalian brain. *J Neurochem* 42:1-11.

Furuta A, Rothstein JD, Martin LJ (1997) Glutamate transporter protein subtypes are expressed differentially during rat CNS development. *J Neurosci* 17:8363-8375.

Gibson CJ (1992) Overview of spinal cord injury. *Phys Med Rehab Clin North Am* 3:699-709.

Graf R, Dohmen C, Kumura E, Rosner G, Heiss W-D (1998) Extracellular shifts of GABA, adenosine and ion activities in cortical gray and subcortical white matter during global ischemia in cats. *Soc Neurosci Abstr* 24:983.

Griffiths R, Dunlop J, Gorman A, Senior J, Grieve A (1994) L-trans-pyrrolidine-2,4-dicarboxylate and cis-1-aminocyclobutane-1,3- dicarboxylate behave as transportable, competitive inhibitors of the high-affinity glutamate transporters. *Biochem Pharmacol* 47:267-74.

Imaizumi T, Kocsis JD, Waxman SG (1997) Anoxic injury in the rat spinal cord - pharmacological evidence for multiple steps in Ca^{2+} -dependent injury of the dorsal columns. *J Neurotrauma* 14:299-311.

Kriegler S, Chiu SY (1993) Calcium signaling of glial cells along mammalian axons. *J Neurosci* 13:4229-4245.

Leppanen LL, Stys PK (1997) Ion transport and membrane potential in CNS myelinated axons. II: effects of metabolic inhibition. *J Neurophysiol* 78:2095-2107.

Levy LM, Warr O, Attwell D (1998) Stoichiometry of the glial glutamate transporter GLT-1 expressed inducibly in a chinese hamster ovary cell line selected for low endogenous Na⁺-dependent glutamate uptake. *J Neurosci* 18:9620-8.

LoPachin RM, Stys PK (1995) Elemental composition and water content of rat optic nerve myelinated axons and glial cells: effects of in vitro anoxia and reoxygenation. *J Neurosci* 15:6735-6746.

Matsuo A, Lee GC, Terai K, Takami K, Hickey WF, McGeer EG, McGeer PL (1997) Unmasking of an unusual myelin basic protein epitope during the process of myelin degeneration in humans: a potential mechanism for the generation of autoantigens. *Am J Pathol* 150:1253-1266.

Matute C, Sanchez-Gomez MV, Martinez-Millan L, Miledi R (1997) Glutamate receptor-mediated toxicity in optic nerve oligodendrocytes. *Proc Natl Acad Sci U S A* 94:8830-8835.

Maxwell WL, Povlishock JT, Graham DL (1997) A mechanistic analysis of nondisruptive axonal injury: a review. *J Neurotrauma* 14:419-440.

McDonald JW, Althomsons SP, Hyrc KL, Choi DW, Goldberg MP (1998) Oligodendrocytes from forebrain are highly vulnerable to AMPA/kainate receptor-mediated excitotoxicity. *Nat Med* 4:291-297.

Mealing GA, Lanthorn TH, Murray CL, Small DL, Morley P (1999) Differences in degree of trapping of low-affinity uncompetitive N-methyl-D-aspartic acid receptor antagonists with similar kinetics of block. *J Pharmacol Exp Ther* 288:204-10.

Mennerick S, Dhond RP, Benz A, Xu W, Rothstein JD, Danbolt NC, Isenberg KE, Zorumski CF (1998) Neuronal expression of the glutamate transporter GLT-1 in hippocampal microcultures. *J Neurosci* 18:4490-4499.

Paternain AV, Morales M, Lerma J (1995) Selective antagonism of AMPA receptors unmasks kainate receptor-mediated responses in hippocampal neurons. *Neuron* 14:185-9.

Povlishock JT, Christman CW (1995) Diffuse axonal injury. In: *The Axon: Structure, Function and Pathophysiology* (S. G. Waxman, J. D. Kocsis and P. K. Stys ed), pp 504-529. New York: Oxford University Press.

Ransom BR, Walz W, Davis PK, Carlini WG (1992) Anoxia-induced changes in extracellular K⁺ and pH in mammalian central white matter. *J Cereb Blood Flow Metab* 12:593-602.

Ransom BR, Waxman SG, Davis PK (1990) Anoxic injury of CNS white matter: protective effect of ketamine. *Neurology* 40:1399-1403.

Roettger V, Lipton P (1996) Mechanism of glutamate release from rat hippocampal slices during in vitro ischemia. *Neuroscience* 75:677-85.

Rose CR, Waxman SG, Ransom BR (1998) Effects of glucose deprivation, chemical hypoxia, and simulated ischemia on Na⁺ homeostasis in rat spinal cord astrocytes. *J Neurosci* 18:3554-3562.

Rosenberg LJ, Teng YD, Wrathall JR (1999) 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(f)quinoxaline reduces glial loss and acute white matter pathology after experimental spinal cord contusion. *J Neurosci* 19:464-475.

Rothstein JD, Martin L, Levey AI, Dykes-Hoberg M, Jin L, Wu D, Nash N, Kuncl RW (1994) Localization of neuronal and glial glutamate transporters. *Neuron* 13:713-25.

Rutledge EM, Aschner M, Kimelberg HK (1998) Pharmacological characterization of swelling-induced D-[3H]aspartate release from primary astrocyte cultures. *Am J Physiol* 274:C1511-20.

Schmitt A, Asan E, Puschel B, Jons T, Kugler P (1996) Expression of the glutamate transporter GLT1 in neural cells of the rat central nervous system: non-radioactive in situ hybridization and comparative immunocytochemistry. *Neuroscience* 71:989-1004.

Stys PK (1994) WaveTrak: a data acquisition system and waveform database for the Macintosh. *Sci. Comput. Automation* 10:19-24.

Stys PK, LoPachin RM (1998) Mechanisms of ion flux in anoxic myelinated CNS axons. *Neuroscience* 82:21-32.

Stys PK, Waxman SG, Ransom BR (1992) Ionic mechanisms of anoxic injury in mammalian CNS white matter: role of Na⁺ channels and Na⁺-Ca²⁺ exchanger. *J Neurosci* 12:430-439.

Sutherland ML, Delaney TA, Noebels JL (1996) Glutamate transporter mRNA expression in proliferative zones of the developing and adult murine CNS. *J Neurosci* 16:2191-207.

Tator CH, Koyanagi I (1997) Vascular mechanisms in the pathophysiology of human spinal cord injury. *J Neurosurg* 86:483-492.

Teng YD, Wrathall JR (1997) Local blockade of sodium channels by tetrodotoxin ameliorates tissue loss and long-term functional deficits resulting from experimental spinal cord injury. *J Neurosci* 17:4359-4366.

Trapp BD, Bernier L, Andrews SB, Colman DR (1988) Cellular and subcellular distribution of 2',3'-cyclic nucleotide 3'-phosphodiesterase and its mRNA in the rat central nervous system. *J Neurochem* 51:859-68.

Wrathall JR, Choiniere D, Teng YD (1994) Dose-dependent reduction of tissue loss and functional impairment after spinal cord trauma with the AMPA/kainate antagonist NBQX. *J Neurosci* 14:6598-607.

Wrathall JR, Teng YD, Marriott R (1997) Delayed antagonism of AMPA/kainate receptors reduces long-term functional deficits resulting from spinal cord trauma. *Exp Neurol* 145: 565-573.

Zerangue N, Kavanaugh MP (1996) Flux coupling in a neuronal glutamate transporter. *Nature* 383:634-7.

Figure 1 A: schematic of recording arrangement. Dorsal column slices were incubated in an *in vitro* recording chamber. Stimulating and recording surface electrodes were used to evoke compound propagated action potentials. B: Representative tracings of compound action potentials shown at intervals of 1 hr recorded over 3 hrs demonstrating the stability of the shape and amplitude, even with repositioning of the electrodes to allow study of multiple slices during the same experiment (see Methods). C: Graph quantitatively showing stable peak amplitudes over 3 hrs at 37 °C.

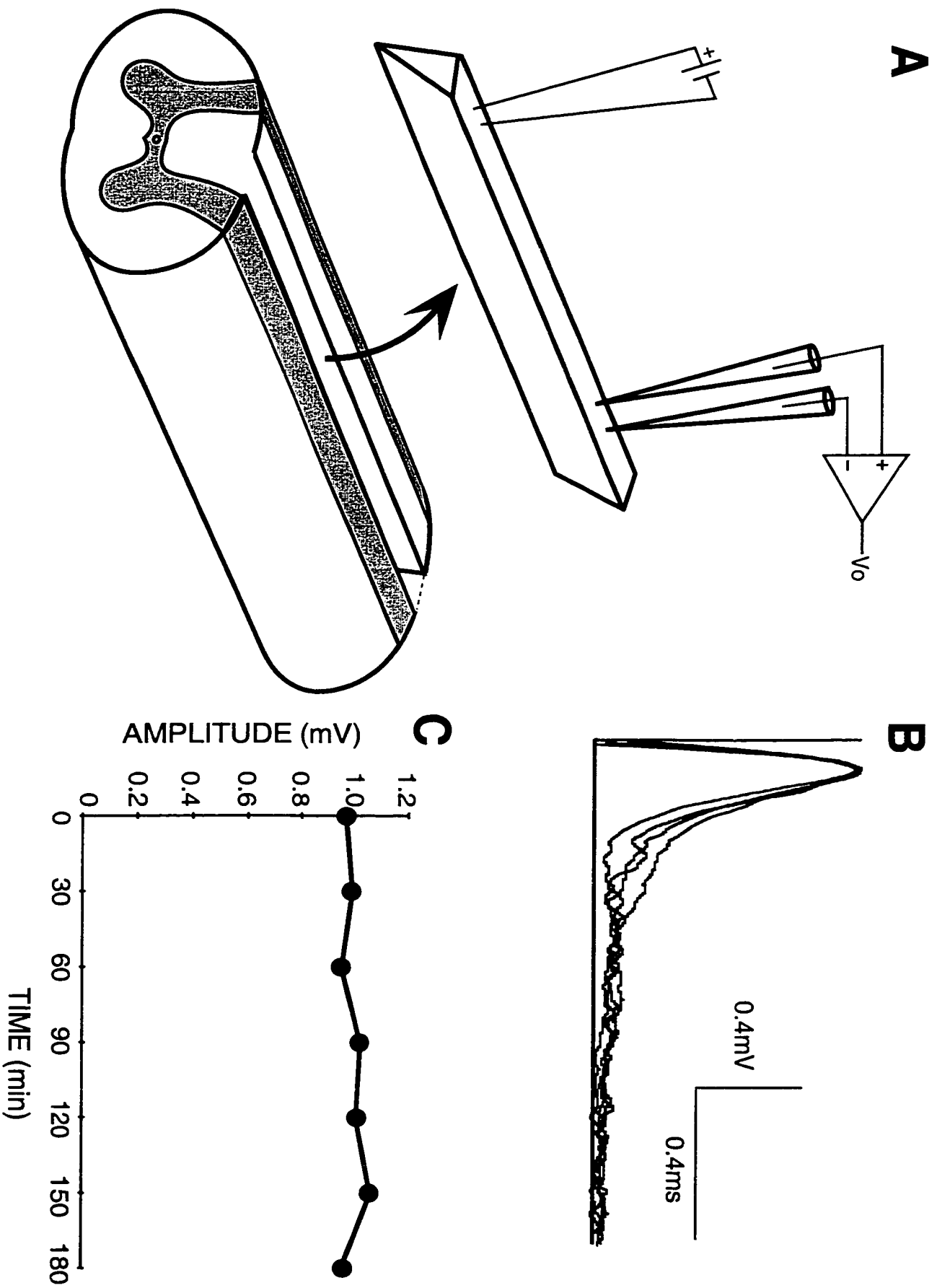


Figure 1

Figure 2 Bar graphs illustrating recovery of compound action potential amplitudes recorded from dorsal column slices *in vitro* following 60 min of anoxia (A) or 15 s of traumatic compression at 2 g (C). Peak amplitudes were normalized to baseline responses recorded at time 0 (see Methods). Drugs were applied beginning 60 min before and continued until 15 min after injury (anoxia or trauma). Slices were exposed to 60 min of anoxia, and compound action potentials measured at 60 and 120 min of reoxygenation (60' and 120' *post-anoxia* bars). In aCSF alone, amplitudes recovered to $\approx 27\%$ of pre-anoxic control (*ctrl anoxia*, n=12), whereas recovery was significantly enhanced to $\approx 55\%$ of control by kynurenic acid (1 mM, 'KYN', n=6) or GYKI52466 (30 μ M, 'GYKI', n=7) (panel A). Similarly, GYKI52466 (n=14) improved recovery after trauma from 35% (*ctrl SCI*, n=13) to 65% of pre-injury control amplitude. Panels B and D show representative compound action potential tracings obtained following anoxia or trauma. These data indicate that endogenous glutamate contributes to functional injury of isolated dorsal columns during anoxia and trauma, acting mainly through AMPA receptors. * $p < 0.01$ compared to time-matched readings of slices injured in the absence of drug.

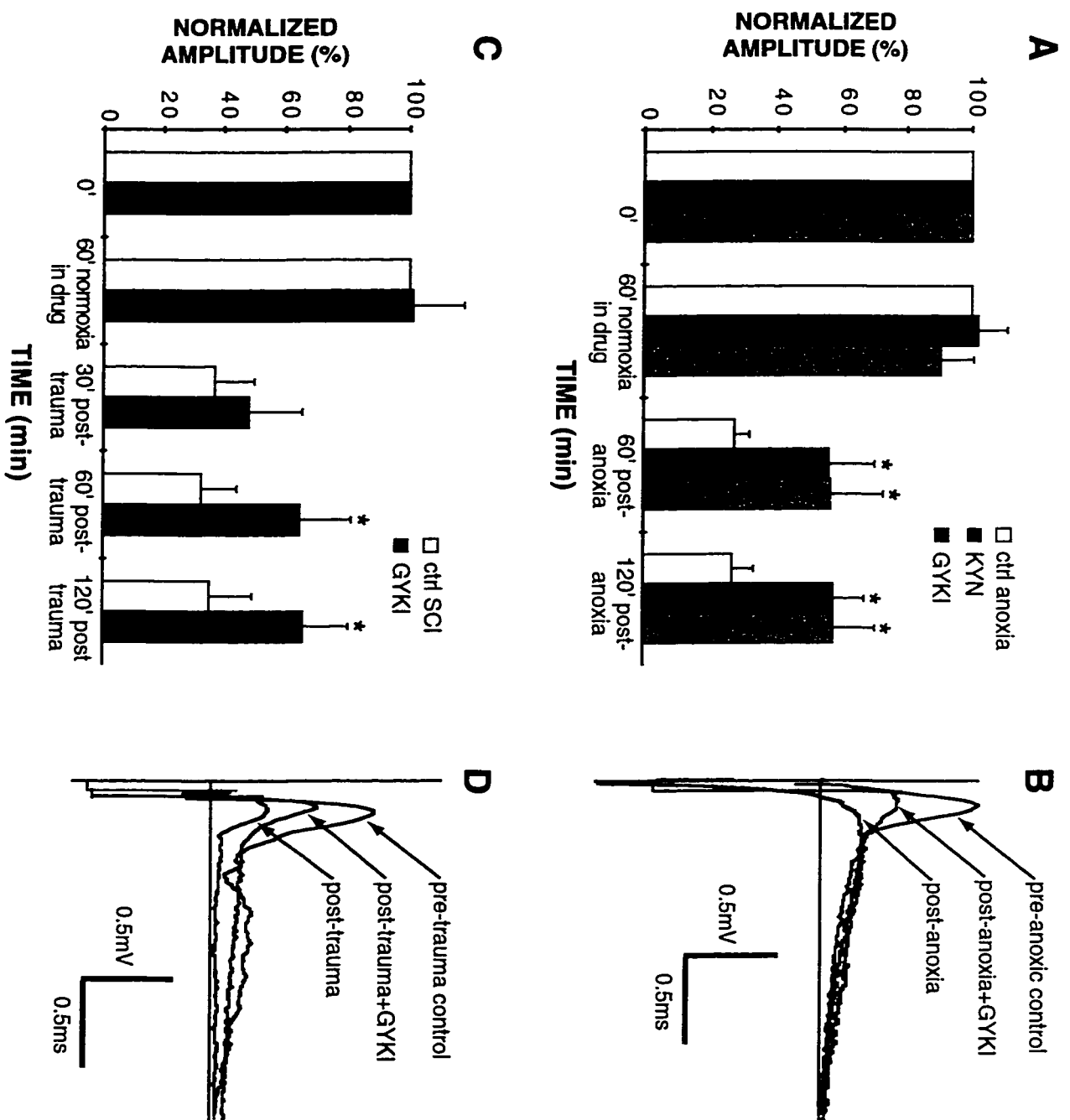


Figure 2

Figure 3 Immunohistochemistry of dorsal column axons stained for neurofilament (green) outlining axon cylinders, and damaged myelin detected by antiserum raised against degenerated myelin basic protein (red). *A*: control sections show virtually no myelin damage. A 60 min anoxic exposure caused significant myelin damage as shown by strong immunoreactivity surrounding many axon cylinders (arrowheads, panels *B* and *C*). Panel *C* shows a higher power view of a single damaged axon. The AMPA receptor blocker GYKI52466 greatly reduced the degree of anoxic myelin damage (*D* and *E*). Sections from the injury focus showed that traumatic compression ('*SCI*') also resulted in damage to myelin (red signal, panel *F*) as well as disruption of axon cylinders as evidenced by distorted neurofilament profiles. Trauma in the presence of the Na⁺-dependent glutamate transport inhibitor L-trans-pyrrolidine-2,4-dicarboxylic acid ('*SCI* + *PDC*') significantly reduced myelin injury (panel *G*). Bars 10 μm.

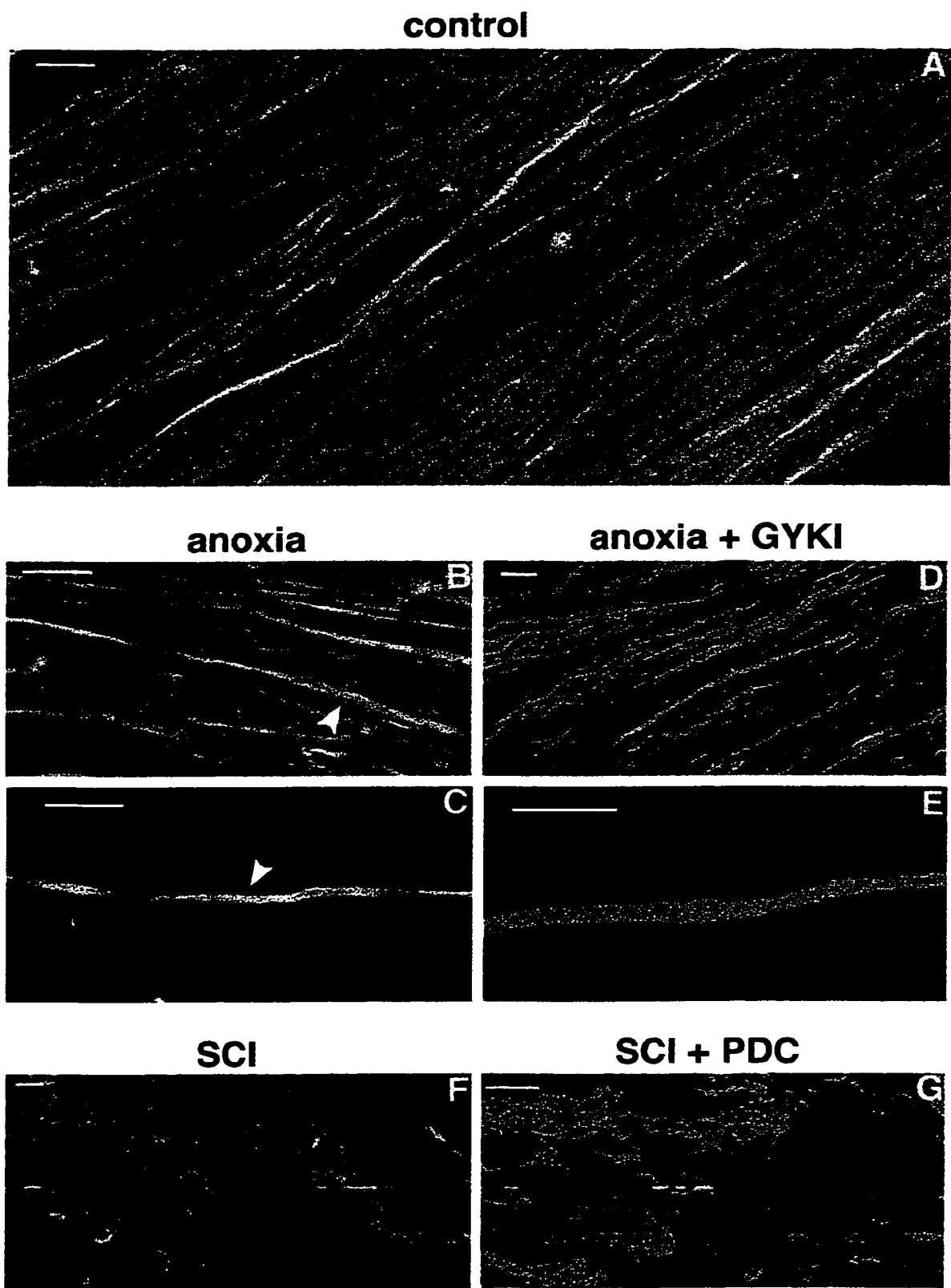


Figure 3

Figure 4 Effect of Na⁺-dependent glutamate transport inhibitors on the recovery of compound action potentials following anoxia or trauma. Drugs were applied beginning 60 min before and continued until 15 min after injury. Neither inhibitor (dihydrokainate [DHK] or L-trans-pyrrolidine-2,4-dicarboxylic acid [PDC], both 1 mM) had any significant effect on pre-injury responses (60' normoxia in drug bars). Both agents improved compound action potential amplitudes significantly following 60 min of anoxia (A) or a 15 s traumatic compression (B). * p < 0.01 compared to time-matched readings of slices injured in the absence of drug. Panels C and D show representative compound action potential tracings. Bar graph in panel E summarizes semiquantitative confocal glutamate immunofluorescence results in three intracellular compartments. Anoxia caused significant depletion of cytosolic glutamate in axon cylinders and oligodendrocytes, but not astrocytes; this depletion was completely prevented by PDC (* p < 0.01, ** p < 0.05). These results indicate that endogenous glutamate is released by reverse operation of Na⁺-dependent glutamate transporters during anoxic or traumatic injury. (n's: panel A, ctrl anoxia:12, DHK:7, PDC:7; panel B, ctrl SCI:13, PDC:7; panel E: minimum of 10 images, each containing multiple regions of interest per group).

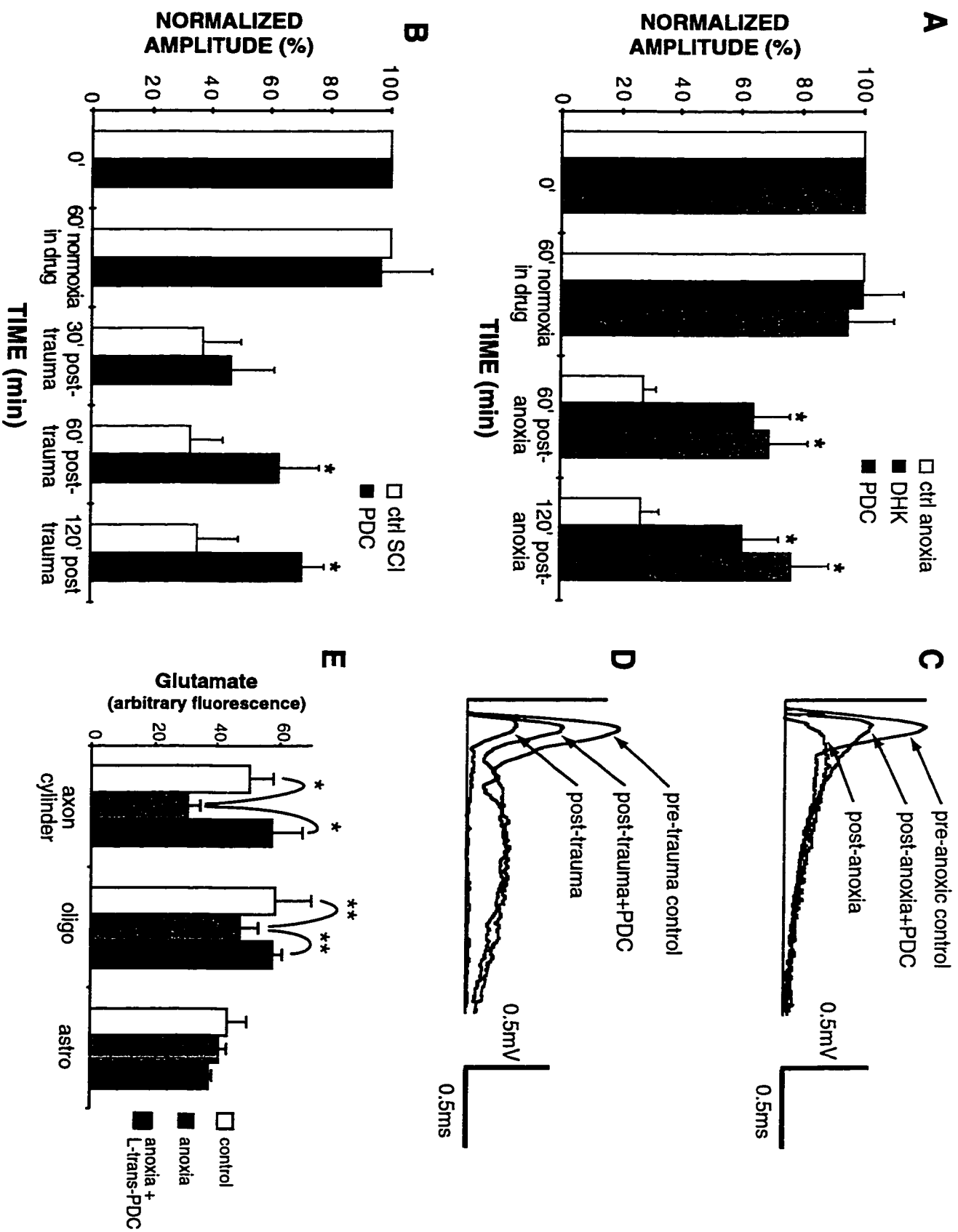


Figure 4

Figure 5 Confocal microscopic images of dorsal columns showing representative immunohistochemistry of three isoforms of glutamate transporter (red), double stained with standard markers (green). *A.* Individual myelinated axon showing faint GLT1 signal within the axon cylinder, but no detectable stain within the myelin sheath outlined using anti-myelin basic protein (MBP) antibodies. *B.* GLT1 was present at high density in cell bodies and processes of astrocytes stained with GFAP resulting in yellow signal indicating colocalization of these two proteins. *C.* Single myelinated axon showing GLAST signal throughout the full thickness of the myelin sheath, with stain within the axon cylinder itself in some fibers. *D.* GLAST and GFAP colocalized in all GFAP-positive astroglia. *E.* The EAAC1 isoform did not localize to the myelin sheath, nor was it convincingly found within the axon cylinders. *F.* GFAP-positive astrocytes occasionally displayed EAAC1 immunoreactivity which was much less consistent than with GLT1 and GLAST. There was considerable intervening stain outside myelin and astrocytic regions (see Results). Bars 10 μm .

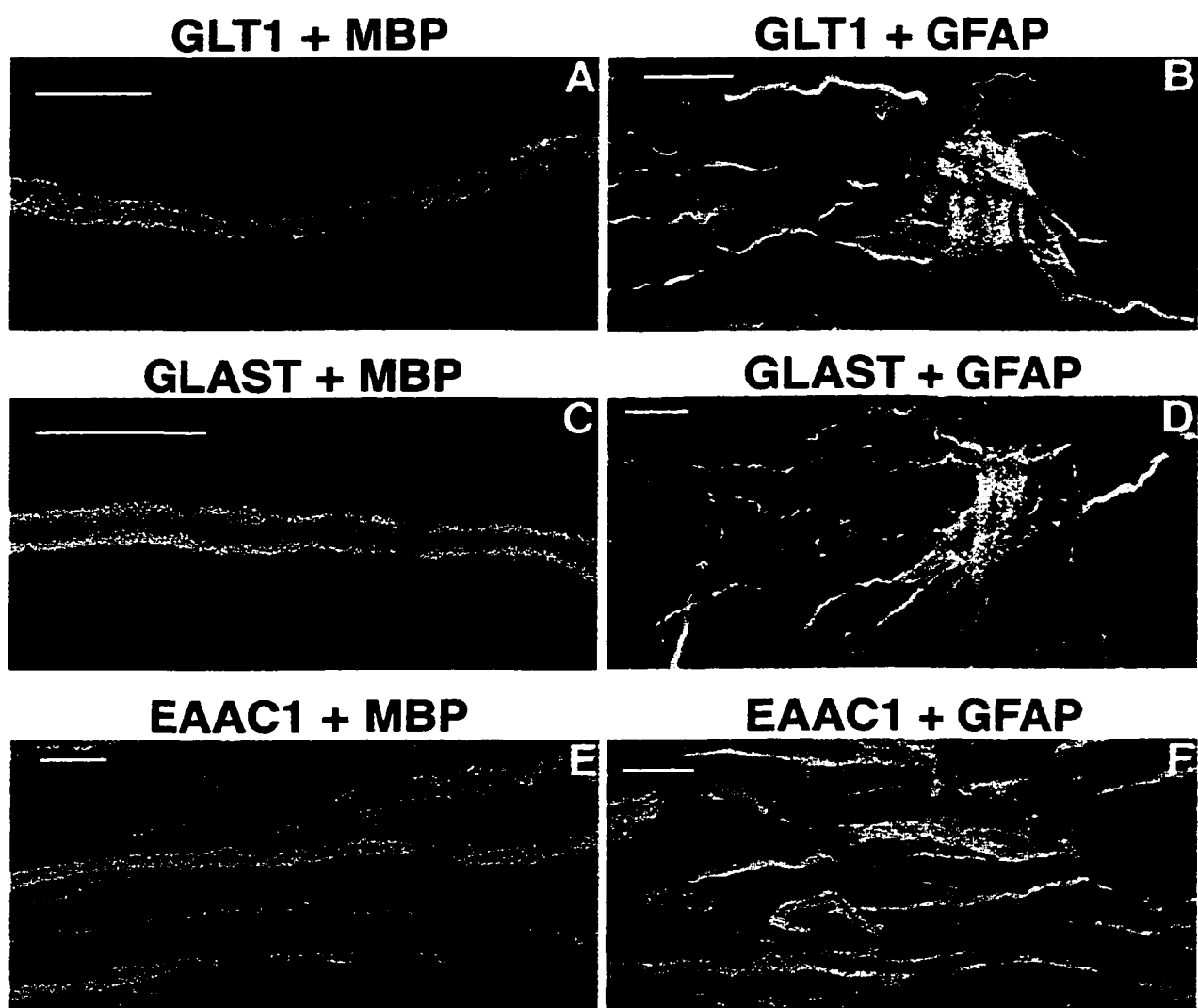


Figure 5

Chapter 5

General Discussion

Disruption of white matter tracts plays a pivotal role in causing severe and widespread disability in several disorders, such as spinal cord injury (SCI) and multiple sclerosis, and is in large part due to the functional interruption of myelinated axons, i.e., the slowing or failure in transmitting information to and from neurons in the brain and spinal cord. Current treatments for these diseases are still very limited, but recent progress in understanding of cellular and molecular mechanisms underlying central nervous system (CNS) axonal dysfunction provides promise for improving the functional recovery of this unique structure. Blockade of Na⁺ entry via voltage-gated Na⁺ channels has been shown to be protective against *in vitro* (Agrawal and Fehlings, 1996; Imaizumi et al., 1997; Stys, 1998; Stys et al., 1995; Stys et al., 1992a) and *in vivo* anoxic/traumatic axonal injury (Rosenberg et al., 1999b; Teng and Wrathall, 1997). Inhibition of excitotoxic mechanisms involving AMPA/kainate receptors has been shown to confer both functional and structural benefits after *in vitro* (Agrawal and Fehlings, 1997) and *in vivo* SCI (Rosenberg et al., 1999a; Wrathall et al., 1992; Wrathall et al., 1994). In this study, we report novel findings by systematically investigating the precise cellular mechanisms of glutamate toxicity, and equally importantly, the glutamate release mechanisms in CNS white matter tissue. The improved understanding of the fundamental mechanisms underlying anoxic/ischemic or traumatic white matter dysfunction will be important for guiding the rational design of neuroprotectants in diseases involving myelinated axons.

5.1 Principal findings in the present studies

5.1.1 Glial components in dorsal column are vulnerable to excitotoxicity via AMPA receptors

Using combined techniques including electrophysiology, pharmacology and immunohistochemistry, we demonstrate that the myelinated axons in dorsal columns are vulnerable to irreversible excitotoxic injury primarily dependent on AMPA receptor activation triggering Ca^{2+} influx through the Ca^{2+} -permeable AMPA ion channels. The cellular targets damaged by excitotoxins include oligodendrocytes, astrocytes as well as myelin sheath, consistent with the distribution of GluR3 and GluR4 in the first two cell types and GluR4 in myelin; GluR2 is notably absent from white matter (Table 1 in *Chapter 4*). By contrast, axon cylinders are spared as indicated by no increase in spectrin breakdown, a marker of cytoskeletal injury. In white matter tracts, myelin, the electrical insulator which prevents current leaks across the axolemma, supports rapid and reliable saltatory conduction along myelinated axons, with damage to this structure causing disruption of signal conduction. Oligodendrocytes are responsible for formation of myelin and maintaining axonal conduction, and injury to this cell will cause demyelination. Astrocytes play a role in maintaining ionic homeostasis and energy supply of axons (Amedee et al., 1997), and loss of their function may produce detrimental effects on surrounding fibers (Sykova et al., 1992). Thus, our finding of glial vulnerability to glutamate in white matter tracts is consistent with the reports that the neuroprotective effects of AMPA/kainate antagonists in models of SCI confer functional protection by sparing glia and myelin (Agrawal and Fehlings, 1997; Rosenberg et al., 1999a; Wrathall et al., 1994; Wrathall et al., 1997).

5.1.2 Collapsed transmembrane ion gradients induce glutamate efflux via reverse Na⁺-dependent transport

The properties of Na⁺-dependent glutamate transporters, which are driven by the transmembrane electrochemical gradients of Na⁺ and K⁺ and membrane potential (Robinson and Dowd, 1997), indicate that glutamate removal from the extracellular space may fail, and the transporter may even operate in a reverse mode causing glutamate release from the cytosol, if the ionic gradients are run down or reversed. This hypothesis has been supported by studies using electrophysiology and pharmacology in cultured cells (Attwell et al., 1993; Gemba et al., 1994; Longuemare and Swanson, 1997; Rutledge and Kimelberg, 1996; Szatkowski et al., 1990; Tachibana and Okada, 1991) or *in situ* gray matter tissue slices (Rossi et al., 2000). In the present study, using *in situ* tissue of isolated spinal cord white matter, we investigated the cellular mechanism of glutamate release by alterations of transmembrane Na⁺ and K⁺ gradients, which have been shown to occur during ischemia/trauma (D'Ambrosio et al., 1999; LoPachin and Stys, 1995; Ransom et al., 1992). We found that internal Na⁺ accumulation induced by inhibiting Na⁺-K⁺-ATPase with ouabain, together with membrane depolarization by high [K⁺]_o, could drive the Na⁺-dependent glutamate transporter to operate in the reverse mode, resulting in glutamate release from axoplasm and subsequent damage to myelinated axons by activation of AMPA receptors. Using confocal microscopy and immunohistochemistry for glutamate, we directly confirmed the release of glutamate, and further demonstrated that levels of this amino acid are significantly depleted in axon cylinders, and to a lesser extent in oligodendrocytes after pump inhibition and high K⁺

depolarization; glutamate levels in astrocytes did not change, however. Our findings suggest that the excitotoxins released through the reverse glutamate transport may contribute to pathogenesis of axonal dysfunction. The protective effect of Na⁺ channel blockade appears to be due in part to interference with this mechanism, unleashed during anoxia/ischemia and trauma in the face of similar ion alterations. For example, *in vitro* exposure of rat optic nerves to anoxia caused early and progressive reduction in axoplasmic K⁺ (to approximately 10% of normal level after 60 min anoxia) in conjunction with an increase in Na⁺ (from 17 to ≈100 mM) in myelinated axons (LoPachin and Stys, 1995). Similarly, compressive injury to spinal cord strips *in vitro* resulted in a four- to five-fold Na⁺ increase and a significant K⁺ decrease in the axoplasm of spinal cord white matter (LoPachin et al., 1999).

5.1.3 Role of glutamate in anoxic/traumatic axonal injury: release via reverse Na⁺-dependent glutamate transport

In white matter tissues subjected to anoxia/ischemia or trauma, the derangements of ionic gradients and membrane potential in injured axons (Agrawal and Fehlings, 1996; Kwo et al., 1989; Leppanen and Stys, 1997; LoPachin and Stys, 1995; LoPachin et al., 1999; Stys and LoPachin, 1996; Stys and LoPachin, 1998) would strongly favor reverse glutamate transport. Using dorsal column injury models subjected to 60 min of anoxia or to a 15 sec mechanical clip compression, our electrophysiological experiments demonstrate that either ionotropic glutamate receptor antagonists, particularly AMPA receptor blockers, or Na⁺-dependent glutamate transport inhibitors significantly protected against anoxia and trauma as shown by improved compound action potentials, indicating a crucial role of

glutamate, released via reverse Na^+ -dependent transport, in the axonal dysfunction dependent on activation of AMPA receptors. Immunohistochemical experiments further showed that oligodendrocytes and, particularly, axon cylinders are the major sources of endogenous glutamate. These findings support the hypothesis that ionic collapses caused by anoxia or trauma are sufficient to reverse Na^+ -dependent glutamate transport, contributing to extracellular glutamate accumulation and axonal conduction failure via damage to the myelin sheath and possibly other structures (for summary see Fig.1). Our novel findings will be very important in designing neuroprotectants for white matter injury since inhibition of glutamate transport appears to be an alternative approach to prevent excitotoxicity of myelinated axons during anoxia or trauma, possibly in addition to directly targeting glutamate receptors with the antagonists. Our results are congruent with recent reports showing that a similar injury mechanism is involved in ischemic injury of gray matter (Rossi et al., 2000) and cultured oligodendrocytes as well (Fern and Moller, 2000).

5.2 Excitotoxicity: white matter vs. gray matter

5.2.1 Ionotropic receptors involving excitotoxic injury

There is overwhelming evidence that glutamate is very toxic to neurons mainly through excessive Ca^{2+} entry via ionotropic glutamate receptors and other routes such as voltage-gated Ca^{2+} channels and reverse Na^+ - Ca^{2+} exchange (Carriedo et al., 1996; Choi and Rothman, 1990; Michaelis, 1998; Ozawa et al., 1998). This excitotoxic mechanism has been shown to underlie the pathogenesis of various CNS insults including anoxia/ischemia and trauma (Bullock and Fujisawa, 1992; Choi and Rothman, 1990;

Dirnagl et al., 1999; Lee et al., 1999; Meldrum, 1994; Meldrum et al., 1992; Smith et al., 1993). The NMDA receptor, an ion channel highly permeable to Ca^{2+} , has been shown to play a pivotal role in neuronal death in ischemia and trauma (Albers et al., 1989; Buchan and Pulsinelli, 1990), which can be prevented by selective antagonism of NMDA receptors (Rothman and Olney, 1995). Subsequent studies found that AMPA/kainate receptors also contribute to neuronal damage during ischemia or trauma, since their antagonists, such as NBQX, YM90K and GYKI52466, are effective in preventing neuronal death due to brain ischemia/trauma (Buchan et al., 1991; Le Peillet et al., 1992; Sheardown et al., 1990; Sheardown et al., 1993; Turski et al., 1998; Yatsugi et al., 1996), consistent with the important role of AMPA receptors in the pathogenesis of neuronal death. Therefore, overstimulation of either NMDA or AMPA/kainate receptors by exogenous or endogenous glutamate, with subsequent Ca^{2+} influx via ion channels, may lead to excessive intracellular Ca^{2+} accumulation and lethal metabolic derangements in neurons. In contrast, in white matter devoid of neuronal somata, glutamate excitotoxicity is principally mediated via Ca^{2+} -permeable AMPA, but not NMDA receptors (*Chapter 2*; Agrawal and Fehlings, 1997; McDonald et al., 1998; Stys and Li, 2000); and more importantly, the cellular targets of injury are glial components, but not the neuronal element, i.e. the axon cylinder (*Chapter 2*; Rosenberg et al., 1999a). Interestingly, one of the most significant characteristics of white matter injury is direct AMPA receptor-mediated damage to myelin sheath itself, which seems to play a critical role in the genesis of rapid conduction failure of myelinated axons during anoxia/ischemia and trauma (*Chapter 4*).

The pivotal role of Ca^{2+} influx via AMPA receptors in excitotoxic injury indicates that the functional properties of AMPA receptor may directly be coupled to cell damage. Generally, the Ca^{2+} permeability and rectification of AMPA receptors are controlled by the presence or absence of the GluR2 subunit (Bochet et al., 1994; Hollmann et al., 1991), with the receptors lacking this subunit being highly permeable to Ca^{2+} (for review see *Chapter 1*). In excitotoxic injury of white matter, direct Ca^{2+} entry through the ion channel pores of AMPA receptors plays a critical role in internal Ca^{2+} accumulation (*Chapter 2*), though other Ca^{2+} routes can not be completely excluded. Reverse transcription-polymerase chain reaction and immunohistochemistry indicate that the GluR2 subunit is absent in myelinated axons (Table in *Chapter 2*; Matute et al., 1997), and application of the selective Ca^{2+} -permeable AMPA receptor blocker Joro spider toxin could largely prevent glutamate excitotoxicity to dorsal column white matter (Fig. 3 in *Chapter 2*). In gray matter, in contrast, the AMPA receptors expressed in most neurons have been shown to be heteromeric complexes containing GluR2, though a small subpopulation of central neurons, such as GABAergic forebrain neurons (Jonas et al., 1994), express Ca^{2+} -permeable AMPA channels (Iino et al., 1990) and are unusually vulnerable to AMPA receptor-mediated injury (Brorson et al., 1994; Turetsky et al., 1994). Therefore, the Ca^{2+} entry mechanisms following AMPA receptor stimulation are relatively complicated in neuronal excitotoxicity. First, in addition to direct permeation of Ca^{2+} ions through AMPA ion channels (Brorson et al., 1994; Carriedo et al., 1996; Turetsky et al., 1994), AMPA receptor activation appears to produce Ca^{2+} -dependent excitotoxicity by indirect means, leading to toxic Ca^{2+} entry via voltage-gated Ca^{2+} channels (Metzger et al., 2000; Savidge and Bristow, 1998) or through Na^+ loading and

subsequent reversal of Na^+ - Ca^{2+} exchange (Hoyt et al., 1998; Itoh et al., 1998). Second, Ca^{2+} ions may enter neurons via AMPA receptors expressing GluR2 (Brorson et al., 1992; Geiger et al., 1995; Washburn et al., 1997). The multimeric receptors expressed in the cells containing GluR2 may allow a fraction of AMPA receptors to lack GluR2 subunits, resulting in large Ca^{2+} fluxes via a small proportion of highly Ca^{2+} -permeable AMPA receptors (Burnashev et al., 1992), or alternatively, even receptors containing GluR2 subunits permit modest Ca^{2+} influx depending on the receptor subunit composition (Brorson et al., 1999). Indeed, intermediate Ca^{2+} permeable AMPA receptors have been observed in various cells, such as some neurons of hippocampus (Isa et al., 1996; Lerma et al., 1994), retina (Zhang et al., 1995), brainstem (Otis et al., 1995), and dorsal spinal cord (Goldstein et al., 1995). Lastly, it has been found that during transient forebrain global ischemia, GluR2 expression is preferentially decreased in CA1 hippocampal neurons (Aronica et al., 1998; Gorter et al., 1997; Pellegrini-Giampietro et al., 1997; Pellegrini-Giampietro et al., 1994; Pellegrini-Giampietro et al., 1992), which usually express poorly Ca^{2+} permeable heteromeric AMPA receptors containing considerable amounts of GluR2 subunits. The increased formation of Ca^{2+} permeable AMPA receptors following ischemia may contribute to delayed neuronal death (Oguro et al., 1999). Taken together, overactivation of AMPA receptors may trigger cellular damage to gray and white matter, but Ca^{2+} influx mechanisms appear to differ partially due to the subunit constitution variations of AMPA receptors in both tissue types.

Notably, though ionotropic glutamate receptors are shown to be central in neural cell death, recent studies suggest that metabotropic glutamate receptors (mGluR) also appear

to contribute to cellular injury of both gray (Allen et al., 1999; Faden et al., 1997; Gong et al., 1995; Nicoletti et al., 1996; Schroder et al., 1999) and white matter (Agrawal et al., 1998) tissues, with activation of mGluR resulting in neurotoxicity or neuroprotection depending on the subtype(s) expressed in the cells. For instance, activation of group I mGluR, leading to internal Ca^{2+} release via G-protein- IP_3 pathway and ryanodine channels (Fagni et al., 2000), contributes to the pathophysiology of ischemic and traumatic tissue damage, but stimulation of group II mGluR may be neuroprotective against ischemic insult through a mechanism of inhibiting the release of excitotoxin (Nicoletti et al., 1996).

5.2.2 Release mechanisms of glutamate in anoxia/ischemia or trauma

The glutamate release mechanism during anoxia, ischemia or trauma is still controversial with a variety of reports showing Ca^{2+} -dependent vesicular release (Drejer et al., 1985; Katayama et al., 1991) or Ca^{2+} -independent non-exocytotic release, primarily through reverse glutamate transport (Bullock et al., 1998; McMahon and Nicholls, 1991; Szatkowski and Attwell, 1994; Takahashi et al., 1997) or anion channels (Bednar et al., 1992). The discrepancies of glutamate release appear to depend on the degrees of energy deficiency and timing of ischemia during the pathogenesis of neuronal injury. At the early stage of ischemia, such as within a few minutes of oxygen deprivation, it is likely that the concentrations of neuronal ATP, though reduced, are still high enough to maintain vesicle docking and glutamate release from synapses through exocytosis (Obrenovitch, 1996; Szatkowski and Attwell, 1994). With oxygen deprivation becoming severe during delayed ischemia, the continuing fall of ATP levels will inhibit exocytotic

glutamate release, but instead, the reduced transmembrane Na^+ and K^+ gradients, together with depolarization (to approximately -20 mV) (Szatkowski and Attwell, 1994), may drive Na^+ -dependent glutamate transporters to run in a backward fashion (Takahashi et al., 1997), thus, releasing toxic levels of glutamate into the extracellular space, rather than taking up this amino acid into cell. Therefore, two phases of glutamate release have been suggested to involve ischemic tissue injury in gray matter: the initial Ca^{2+} -dependent exocytosis from pre-synaptic membranes, and the delayed efflux via reverse Na^+ -dependent glutamate transport. However, in white matter tissues lacking synaptic structures, the Ca^{2+} -independent glutamate release, particularly reversal of Na^+ -dependent glutamate transporter, is probably the principal route for excitotoxin efflux during ischemia/trauma, though the other release pathways, such as swelling-activated anion channels (Basarsky et al., 1999; Kimelberg et al., 1990), could not be ruled out. In the present study, using isolated dorsal column slices, we proved the glutamate release via reverse Na^+ -dependent transport in two ways. First, elevations of intracellular Na^+ with ouabain and external K^+ concentration by addition of KCl, designed to mimic the events of ischemia/trauma, lead to significant AMPA receptor-mediated injury which is largely prevented by inhibition of Na^+ -dependent glutamate transport(*Chapter 3*). Second, using *in vitro* ischemic/traumatic models, we demonstrate that glutamate release from axon cylinders, and to a lesser degree from oligodendrocytes via the same mechanism, contributes significantly to conduction failure (*Chapter 4*). Taken together, glutamate release via both Ca^{2+} -dependent (exocytosis) and Ca^{2+} -independent mechanisms (reverse glutamate transport) contributes to ischemic cell injury of gray matter; in contrast, the latter mechanism, especially the backward operation of Na^+ -dependent glutamate

transporter, appears to be the major release route of excitotoxin in ischemic and traumatic white matter injury *in situ*.

5.3 Axo-glial signaling

At excitatory synapses where Ca^{2+} -permeable AMPA and NMDA receptors are co-expressed, Ca^{2+} inflow through AMPA receptors is comparable to that via NMDA receptors at resting membrane potential because of the block by extracellular Mg^{2+} , suggesting a functional importance of AMPA receptors for excitatory transmission in gray matter (Funk et al., 1995; Jonas and Burnashev, 1995; Otis et al., 1995; Staubli et al., 1994). In white matter tissue, our studies indicate that the glial cells in dorsal columns, including oligodendrocytes, astrocytes and myelin as well, express functional AMPA receptor proteins, particularly GluR3 and GluR4 subunits, and that these receptors play an important role in mediating white matter injury under certain pathologic conditions. However, the physiological functions of these ionotropic glutamate receptors are not well understood. Traditionally, neural glia are thought to be inexcitable due to inability to respond to electrical stimulation with an action potential, but recent investigations indicate that they are not passive, but are actively involved in neural functions in both central and peripheral nervous systems. Glial cells, both in culture and in gray matter *in situ*, are able to respond to a variety of ligands or neuronal firing with changes in intracellular Ca^{2+} and/or membrane potential by activation of the corresponding neurotransmitter receptors, indicating the signaling between soma of neurons and glia coupled by neurotransmitters during neural activation (Cooper, 1995; Dani et al., 1992; Dani and Smith, 1995; Grosche et al., 1999; Pasti et al., 1995; Pasti et

al., 1997; Porter and McCarthy, 1996). In white matter, in addition to formation of myelin, the oligodendrocytes, as well as astrocytes, send longitudinally and radially oriented processes around axons, respectively. The perinodal processes of astrocytes come into close proximity with nodal axolemma to within ~10 nm. Thus, these close anatomical relations between axons and glia, together with expression of functional receptors for glutamate and other neuroactive substances, provide a potential basis for an intimate functional link between these two types of cells, the ligand-mediated axo-glial signaling (Barres, 1991; Steinhauser and Gallo, 1996; Teichberg, 1991; Verkhratsky and Kettenmann, 1996; Verkhratsky et al., 1998).

Indeed, numerous studies support the existence of neurotransmitter-mediated signaling between axons and surrounding glial cells, though most work was principally conducted on non-mammalian axons. The peripheral glia, Schwann cells, generate $[Ca^{2+}]_i$ transients in response to electrical stimulation of peripheral fibers (Jahromi et al., 1992; Lev-Ram and Ellisman, 1995; Reist and Smith, 1992), probably resulting from activity-dependent increase in $[K^+]_o$ (triggering depolarization and opening of VGCC) and activation of AMPA receptors by released glutamate from axons. In frog sciatic nerve, 20-70 Hz stimulation triggered significant Ca^{2+} increase, in the outer Schwann cell cytoplasmic pockets and even in myelin sheath near the nodes of Ranvier (Lev-Ram and Ellisman, 1995). In squid axons, glutamate released from axons during action potentials is able to diffuse across the periaxonal space and activate glutamate receptors on Schwann cells, which in turn trigger the release of acetylcholine from Schwann cells dependent on IP_3 , leading to the activation of cholinergic receptors and the shift of Schwann cell membrane

potential (fast depolarization followed by a prolonged hyperpolarization) (Lieberman and Sanzenbacher, 1992; for review see Chiu and Kriegler, 1994).

In unmyelinated mammalian axonal fiber tracts, receptor-mediated signaling between axons and glial cells has also been demonstrated. Electrical activity of axons facilitates the glial ionotropic and/or metabotropic receptors, triggering the shift of membrane potential and $[Ca^{2+}]_i$ responses in glial cells (Chiu and Kriegler, 1994; Kriegler and Chiu, 1993; Maire et al., 1984). Using neonatal rat optic nerve, a premyelinated axonal fiber tract, Kriegler and Chiu showed that repetitive axonal action potentials (~20Hz) could trigger glial $[Ca^{2+}]_i$ spikes in a frequency-dependent manner, which could be mimicked by activating mGluR directly (Kriegler and Chiu, 1993). Furthermore, these activity-induced glial Ca^{2+} elevations could also be mimicked by altering ionic gradients known to favor glutamate efflux via reversed glutamate transport, suggesting the release of this amino acid from white matter tracts in a non-vesicular fashion during axonal firing. In addition, axonal fibers undergoing repetitive axonal activity have been shown to release adenosine (Chiu and Kriegler, 1994; Maire et al., 1982; Maire et al., 1984), which is capable of modulating glutamate effects by potentiating the role of glutamate through inhibiting glutamate uptake triggered by enhanced arachidonic acid in the presence of protein kinase C (Barbour et al., 1989). Taken together, previous observations support the notion that during repetitive impulses in axonal fiber tracts, axon cylinders are able to send signals to surrounding glia (oligodendrocytes, astrocytes, Schwann cells) by activating glial neurotransmitter receptors (such as glutamate) released in an activity-dependent manner, and reverse operation of transporters elicited by ion alterations during

axonal firing could be an important mechanism for neurotransmitter release. Notably, the structural and functional similarities between myelinated and premyelinated axons suggest the existence of axo-glial signaling in the myelinated axonal tissue, though direct evidence is required. However, as discovered for the first time in the present study, the presence of AMPA receptor GluR4 in the myelin sheath, suggests that axonal activity might directly modulate the metabolism and structure of the sheath itself, independently of, or in addition to, effects from the parent soma.

The significance of axo-glial signaling is not well understood, but this coupling seems to be of great importance in maintaining the integrative functions of axon-glia networks and efficient conduction along axons. Generally, glial cells in axonal fiber tracts are thought primarily to provide structural and metabolic support to axons. The major function of glial Ca^{2+} signaling in axonal fibers appears to provide metabolic coupling between these two cellular components, axon and glia. By sensing the changes in Na^+ and K^+ gradients occurring during repetitive action potentials, the transporters release neurotransmitters (such as glutamate), which act on the corresponding receptors (such as AMPA receptor or mGluR) on glia, and translate the axonal message into Ca^{2+} oscillations. Then, by modulating the levels of glial Ca^{2+} -dependent proteins (protein kinases, phospholipases and cyclic nucleotide), these Ca^{2+} signals result in a variety of metabolic responses (for review see Finkbeiner, 1993). First, glial receptor activation may modulate the excitability of axons by release of neuroactive substances (such as GABA) and by interacting with receptors on axons, such as GABA_A receptors (Sakatani et al., 1991; Saruhashi et al., 1999), consistent with the reports that glial cells can synthesize (Barres,

1991) and release GABA in response to mGluR activation (Gallo et al., 1991). Second, activation of glial receptors may lead to changes in K^+ permeability, contributing to K^+ homeostasis. For example, in rat optic nerve, activation of metabotropic receptors on glia triggers Ca^{2+} -dependent K^+ conductance via second messenger IP_3 (Quandt and MacVicar, 1986). Finally, activation of neurotransmitter receptors, such as AMPA receptors, may have effects on the gene expression in culture and glia *in situ* (Arenander et al., 1989; Mack et al., 1994; Pende et al., 1994).

5.4 Summary of injury mechanisms in white matter and implications for therapy

Recent studies, together with the present investigations, have expanded our understanding of the fundamental pathophysiology involving white matter damage. During anoxia/ischemia or trauma, the energy failure and ion pump impairment will result in collapses of ion transport and transmembrane ion gradients, leading to cell injury of myelinated axons mainly involving two parallel injury pathways. Na^+ accumulation by excessive influx of this ion and attenuated membrane potential will evoke Ca^{2+} overload in axonal cylinders via reverse Na^+ - Ca^{2+} exchange and voltage-gated Ca^{2+} channels. Meanwhile, the reduced transmembrane Na^+ and K^+ gradients and the membrane potential alterations will induce glutamate release through reverse Na^+ -dependent glutamate transporter, leading to Ca^{2+} accumulation in ensheathing myelin and supporting glia by activating AMPA receptors on these cell elements. Thus, during the pathogenesis of anoxic/traumatic cell damage of white matter, axon cylinders suffer a Ca^{2+} -dependent injury primarily mediated by Na^+ - Ca^{2+} exchange and VGCC; whereas glial elements, including the myelin sheath, succumb to injury dependent on an excitotoxic mechanism

involving Ca^{2+} -permeable AMPA receptors (Fig.2). These two parallel pathways constitute the major injury mechanisms of anoxic and traumatic white matter tissues, contributing to the irreversible functional failure of myelinated axons.

Ca^{2+} entry pathways might differ in various cell types, such as axons and glia, but the downstream injury mechanisms appear to be quite similar involving a series of processes including activation of Ca^{2+} -dependent enzymes, generation of free radicals and mitochondrial damage. Many recent studies indicate that the mitochondrion is a key cellular organelle responsible for converting rapid cytoplasmic Ca^{2+} accumulation into cell injury (Kroemer and Reed, 2000; Vergun et al., 1999). Excessive Ca^{2+} entry via glutamate receptors, such as Ca^{2+} -permeable AMPA/kainate channels, or other routes may be accumulated by mitochondria, and the Ca^{2+} uptake by this organelle may cause direct mitochondrial membrane depolarization (Schinder et al., 1996; White and Reynolds, 1996), impairment of energy metabolism (Coyle and Puttfarcken, 1993; Nicholls and Budd, 1998; Wang et al., 1994), and uncoupling of electron transport from ATP production (Beatrice et al., 1980), with resultant release of oxygen free radicals from the electron transport chain (Carriedo et al., 1998; Coyle and Puttfarcken, 1993; Perez Velazquez et al., 1997; Turrens et al., 1985) as well as nitric oxide by activation of Ca^{2+} -dependent NO synthase (Bhardwaj et al., 1997). Thus, following excessive elevation of cytosolic Ca^{2+} , in addition to Ca^{2+} -dependent biochemical pathways, Ca^{2+} overload in mitochondria is an essential process for cell death (Stout et al., 1998). Actually, during the elevated $[\text{Ca}^{2+}]_i$ -mediated insults, there is a positive feedback involving cell damage: mitochondria, an important source of reactive oxygen species, are further impaired by

free-radical-induced disruption of inner membrane and oxidation of various proteins mediating electron transport, H^+ extrusion and ATP production (Kroemer and Reed, 2000). Moreover, mitochondria play a critical role in the genesis of cell apoptosis (Jacobson, 1997; Kroemer and Reed, 2000), which has been shown to play a role in the cell death of glia in spinal cord white matter (Crowe et al., 1997; Li et al., 1999; Liu et al., 1997).

A thorough understanding of the fundamental cellular and molecular mechanisms of tissue injury, together with advances in molecular pharmacology, will guide the logical choice of targets for intervention, and eventually accelerate the design of successful neuroprotective strategies for CNS white matter injury. The advances in excitotoxic injury mechanisms, particularly the novel findings of subcellular injury loci and glutamate release pathway, will allow us to more accurately target injury mechanisms of myelinated axons. Combination of different approaches targeting various mechanisms may yield greater neuroprotection against anoxic and traumatic injury than either approach alone, and may also confer a longer therapeutic window for these disorders. For instance, coapplication of Na^+ channel blockers, mainly targeting axon cylinders (Rosenberg et al., 1999b), with AMPA receptor antagonists, which protect myelin and other glial cells against excitotoxic insult (Fig. 3 in *Chapter 4*, Rosenberg et al., 1999a), may provide superior protection. Alternatively, combinations of the above strategies with others aimed at downstream injury cascades including blocking proteases (Jiang and Stys, 2000; Schumacher et al., 1999; Schumacher et al., 2000), oxygen free radicals, mitochondrial and apoptotic injury pathways, may also yield more promising effects than

any single strategy. It is worth noting that, as found in our study, an alternative approach to efficiently block glutamate toxicity might be achieved by reducing the excitotoxin release, such as through inhibition of Na^+ -dependent transport using the corresponding inhibitors.

5.5 Future directions

5.5.1 Role of Ca^{2+} in glutamate-mediated white matter injury: Ca^{2+} -imaging study

We have shown that glutamate significantly contributes to anoxic and traumatic axonal injury due to reverse glutamate transport (*Chapter 4*) and this glutamate excitotoxicity is Ca^{2+} -dependent and mediated mainly via Ca^{2+} -permeable AMPA receptors (*Chapter 2*). To directly confirm the role of Ca^{2+} in glutamate induced white matter injury and to correlate physiological effects of glutamate with Ca^{2+} changes, it will be interesting to perform experiments to measure the changes of intracellular free Ca^{2+} levels in axons as well as glial components including the myelin sheath in either glutamate (or its agonists) loaded normal dorsal column slices or anoxic/traumatic injured slices with confocal microscopy and electron probe microanalysis (LoPachin and Stys, 1995). In addition, Ca^{2+} imaging can also be performed during modulation of the ionic milieu, such as reduced Na^+ and K^+ gradients, membrane depolarization. To dissect out Ca^{2+} entry pathways, pharmacological experiments can be performed using glutamate receptor antagonists, glutamate transport inhibitors and blockers for Na^+ - Ca^{2+} exchange or voltage gated Ca^{2+} channels.

5.5.2 Neuroprotection of combined agents

Given the complex injury mechanisms in white matter, it appears that only a combined approach aimed at several different molecular targets would be more successful in developing an effective therapeutic strategy for the disorders affecting white matter. Previous work has demonstrated the utility of Na⁺ channel inhibitors as neuroprotective agents in anoxic optic nerve *in vitro* (Stys, 1995; Stys et al., 1992a), *in situ* (Stys and Lesiuk, 1996), anoxic dorsal columns *in vitro* (Imaizumi et al., 1997) and traumatic SCI both *in vitro* (Agrawal and Fehlings, 1996) and *in vivo* (Teng and Wrathall, 1997) by mainly targeting axon cylinders (Rosenberg et al., 1999b). Most recent studies indicate that block of glutamate receptors (particularly AMPA receptors) or Na⁺-dependent transport is also highly protective to anoxic/traumatic white matter injury (*Chapter 4*; Agrawal and Fehlings, 1997; Wrathall et al., 1994) through preventing oligodendrocytes, astrocytes as well as myelin from excitotoxic injury (*Chapter 2*; Rosenberg et al., 1999a). It is likely that neither abnormal Na⁺ influx nor glutamate release will be completely eliminated by its respective inhibitors, but intercepting the injury cascades at two distinct points may be more beneficial than single therapy aimed at each target individually. Therefore, we propose both voltage-gated Na⁺ channel and glutamate receptor/transporter as targets for neuroprotectants.

5.5.3 Neuroprotective studies in a model of *in vivo* SCI

The present project was principally designed to explore the subcellular targets of glutamate-mediated tissue injury and the non-vesicular release of glutamate via reversal of Na⁺-dependent glutamate transporter in spinal cord white matter, but our ultimate goal is to design rational therapy aimed at the above-mentioned mechanisms through inhibiting the deleterious effects of glutamate release and other pathologic processes causing irreversible

tissue injury. In the present studies, we chose to apply *in vitro* models since these are far more easily controlled and amenable to interpretation. To further validate our findings in a more realistic paradigm and to provide more information for clinically relevant pharmacotherapy, it would be very interesting to perform experiments moving to a more realistic and complex *in vivo* model, such as clip compression SCI (Rivlin and Tator, 1978), which more closely resembles clinical traumatic injury of spinal cord. In the complicated *in vivo* model, some issues including toxicity, absorption, CNS penetration of tested agents can be better addressed before these pharmacological drugs could be successful in human SCI. Based on the information obtained from our *in vitro* work, inhibitors of glutamate transport, such as L-trans-pyrrolidine-2,4-dicarboxylate, can be tested in a *in vivo* SCI model. If results are favorable, experiments using combinations of the best Na⁺ channel blockers and glutamate transport inhibitors can be carried out. We predict that such an approach will greatly accelerate our understanding of injury mechanisms to white matter tracts in SCI and the rational development of neuroprotective strategies. In addition, mutant mice deficient in either glutamate receptors or glutamate transporters could also be used as an alternative tool to probe excitotoxic injury mechanism in the CNS white matter (Morikawa et al., 1998; Rothstein et al., 1996).

5.5.4 Axo-glial signaling in myelinated axons

Oligodendroglia, astrocytes and myelin express a variety of receptors for neurotransmitter (*Chapter 2*; Gallo and Russell, 1995; Steinhauser and Gallo, 1996; Teichberg, 1991; Verkhratsky et al., 1998)), but the functional significance of these receptors is far from clear. Previous work suggests the existence of activity-dependent

axonal-to-glial signaling mediated by neuroactive substances in invertebrate peripheral (Evans et al., 1991; Lieberman, 1991) and vertebrate unmyelinated central axons (Kriegler and Chiu, 1993; Wachtler et al., 1998). However, the potentially very important issue of axo-glial signaling in myelinated axons of the mature mammalian brain and spinal cord, with fundamental implications for the physiology and pathophysiology of the CNS, has not been investigated. Our data demonstrate the presence of ionotropic AMPA receptors in the myelin sheath of CNS white matter (*Chapter 2*), indicating that myelin itself may directly respond to axonal firing. However, it is not known whether axonal activity results in corresponding response in this unique structure. More importantly, the precise cellular mechanisms, particularly the neurotransmitters coupling axons and glia, and activity-triggered neurotransmitter release mechanism in fiber tracts, remain to be answered. Thus, it would be very important to observe the signal coupling between axons and surrounding glial cells (including myelin) in myelinated axons with combined techniques such as electrophysiology, pharmacology, Ca^{2+} imaging and confocal microscopy. Investigations on this topic will provide very important information on understanding cellular mechanisms involving glial $[\text{Ca}^{2+}]_i$ signals, $[\text{Ca}^{2+}]_i$ homeostasis and role of glia in maintaining integrative function of axo-glial networks.

Figure 1 Diagram illustrating glutamate release via reverse Na^+ -dependent glutamate transport in white matter. During anoxia/trauma, the attenuated transmembrane Na^+ and K^+ gradients plus membrane depolarization induce the release of glutamate from intracellular compartments through Na^+ -dependent transporter operating in a reverse mode. The excessive efflux of glutamate results in the impairment of the myelin sheath, and possibly also of other supporting glial cells such as oligodendrocytes and astrocytes, via overactivation of ionotropic AMPA receptors.

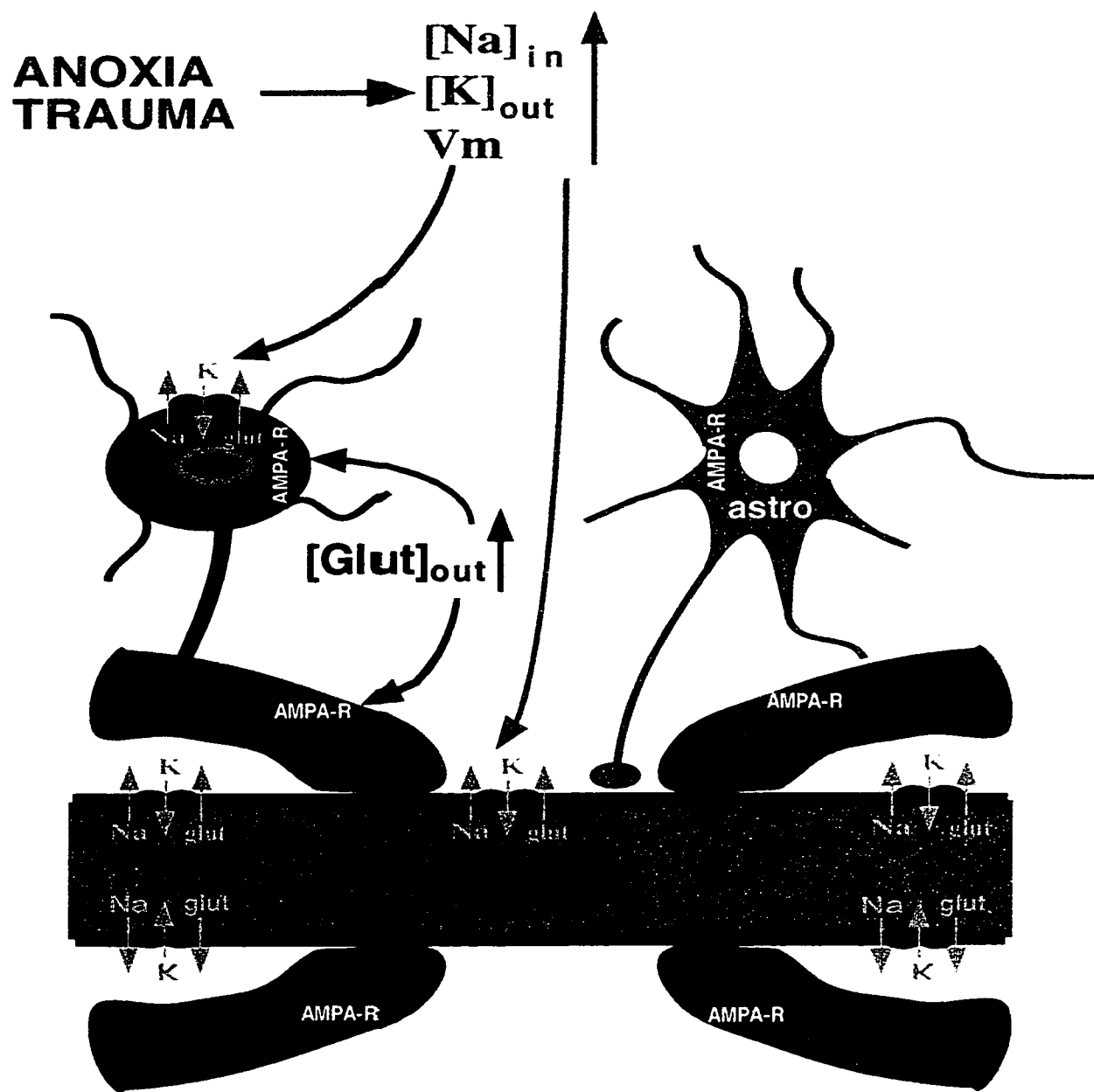


Figure 1

Figure 2 Illustration of anoxic/traumatic injury mechanisms of myelinated axons. Interruption of oxygen and/or glucose supply leads to depletion of ATP stores, evoking failure of ATP-dependent pumps including $\text{Na}^+\text{-K}^+\text{-ATPase}$ and accumulation of internal Na^+ and external K^+ plus depolarized membrane potential. The rise of Na^+ in intracellular compartments, coupled with depolarization induced by K^+ efflux, triggers two parallel injury pathways: 1) Ca^{2+} influx into axon cylinder through reverse $\text{Na}^+\text{-Ca}^{2+}$ exchanger and voltage-gated Ca^{2+} channel (light green); 2) Ca^{2+} overload in glial cells and myelin sheath involving overactivation of AMPA receptors by axoplasmic glutamate released via reverse Na^+ -dependent glutamate transporters (pink). The Ca^{2+} overload in intracellular compartments results in structural and functional injury to axon cylinders, and to glial cells as well, via the downstream injury mechanisms involving excess activation of Ca^{2+} -dependent biochemical pathways, mitochondrial damage and generation of harmful amounts of free radicals.

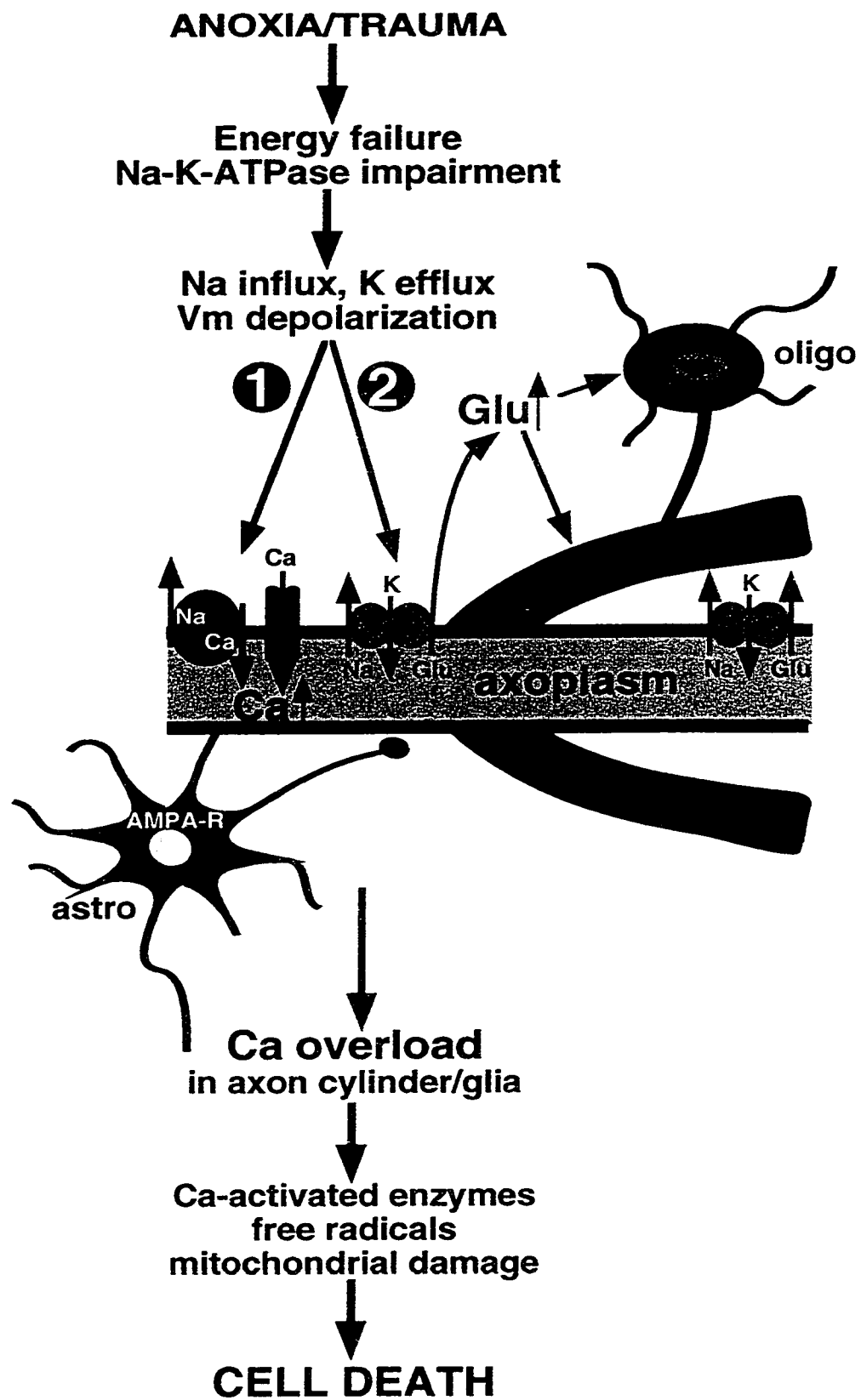


Figure 2

References for General Introduction and Discussion

Abe Y, Yamamoto T, Sugiyama Y, Watanabe T, Saito N, Kayama H, Kumagai T (1999) Apoptotic cells associated with Wallerian degeneration after experimental spinal cord injury: a possible mechanism of oligodendroglial death . J Neurotrauma 16: 945-52.

Adachi M, Takahashi K, Yuge K, Nishikawa M, Miki H, Uyama M (1996) Treatment with bifemelane for optic nerve damage following high intraocular pressure in rat eyes. Eur J Ophthalmol 6: 415-20.

Agrawal SK, Fehlings MG (1996) Mechanisms of secondary injury to spinal cord axons in vitro - role of Na^+ , Na^+ - K^+ -ATPase, the Na^+ - H^+ exchanger, and the Na^+ - Ca^{2+} exchanger. J Neurosci 16: 545-552.

Agrawal SK, Fehlings MG (1997) Role of NMDA and non-NMDA ionotropic glutamate receptors in traumatic spinal cord axonal injury. J Neurosci 17: 1055-1063.

Agrawal SK, Fehlings MG (1998) Role of calcium channels in traumatic spinal cord white matter injury. Soc Neurosci Abstr 24: 743.

Agrawal SK, Theriault E, Fehlings MG (1998) Role of group I metabotropic glutamate receptors in traumatic spinal cord white matter injury. J Neurotrauma 15: 929-41.

Albers GW, Goldberg MP, Choi DW (1989) N-methyl-D-aspartate antagonists: ready for clinical trial in brain ischemia? *Ann Neurol* 25: 398-403.

Allen JW, Ivanova SA, Fan L, Espey MG, Basile AS, Faden AI (1999) Group III metabotropic glutamate receptor activation attenuates traumatic neuronal injury and improves neurological recovery after traumatic brain injury. *J Pharmacol Exp Ther* 290: 112-20.

Amedee T, Robert A, Coles JA (1997) Potassium homeostasis and glial energy metabolism. *Glia* 21: 46-55.

Arai A, Kessler M, Ambros-Ingerson J, Quan A, Yigiter E, Rogers G, Lynch G (1996) Effects of a centrally active benzoylpyrrolidine drug on AMPA receptor kinetics. *Neuroscience* 75: 573-85.

Arenander AT, de Vellis J, Herschman HR (1989) Induction of c-fos and TIS genes in cultured rat astrocytes by neurotransmitters. *J Neurosci Res* 24: 107-14.

Aronica EM, Gorter JA, Grooms S, Kessler JA, Bennett MV, Zukin RS, Rosenbaum DM (1998) Intracarboxylic acid prevents GLUR2 mRNA down-regulation and delayed neurodegeneration in hippocampal CA1 neurons of gerbil after global ischemia. *Proc Natl Acad Sci U S A* 95: 7115-20.

Arriza JL, Eliasof S, Kavanaugh MP, Amara SG (1997) Excitatory amino acid transporter 5, a retinal glutamate transporter coupled to a chloride conductance. *Proc Natl Acad Sci U S A* 94: 4155-60.

Arriza JL, Fairman WA, Wadiche JI, Murdoch GH, Kavanaugh MP, Amara SG (1994) Functional comparisons of three glutamate transporter subtypes cloned from human motor cortex. *J Neurosci* 14: 5559-69.

Attwell D, Barbour B, Szatkowski M (1993) Nonvesicular release of neurotransmitter. *Neuron* 11: 401-7.

Babcock DF, Hille B (1998) Mitochondrial oversight of cellular Ca^{2+} signaling. *Curr Opin Neurobiol* 8: 398-404.

Ballerini L, Bracci E, Nistri A (1995) Desensitization of AMPA receptors limits the amplitude of EPSPs and the excitability of motoneurons of the rat isolated spinal cord. *Eur J Neurosci* 7: 1229-34.

Barbour B, Szatkowski M, Ingledew N, Attwell D (1989) Arachidonic acid induces a prolonged inhibition of glutamate uptake into glial cells. *Nature* 342: 918-20.

Barres BA (1991) New roles for glia. *J Neurosci* 11: 3685-94.

Barres BA, Chun LL, Corey DP (1988) Ion channel expression by white matter glia: I. Type 2 astrocytes and oligodendrocytes. *Glia* 1: 10-30.

Basarsky TA, Feighan D, MacVicar BA (1999) Glutamate release through volume-activated channels during spreading depression. *J Neurosci* 19: 6439-45.

Beatrice MC, Palmer JW, Pfeiffer DR (1980) The relationship between mitochondrial membrane permeability, membrane potential, and the retention of Ca^{2+} by mitochondria. *J Biol Chem* 255: 8663-71.

Bednar MM, Kohut JJ, Kimelberg HK, Gross JJ, Gross CE (1992) In vitro evidence supporting two mechanisms of action for the anion transport inhibitor L-644,711 in cerebral ischaemia. *Neurol Res* 14: 53-6.

Benke TA, Luthi A, Isaac JT, Collingridge GL (1998) Modulation of AMPA receptor unitary conductance by synaptic activity. *Nature* 393: 793-7.

Berger T (1995) AMPA-type glutamate receptors in glial precursor cells of the rat corpus callosum: ionic and pharmacological properties. *Glia* 14: 101-14.

Bhardwaj A, Northington FJ, Ichord RN, Hanley DF, Traystman RJ, Koehler RC (1997) Characterization of ionotropic glutamate receptor-mediated nitric oxide production in vivo in rats. *Stroke* 28: 850-6; discussion 856-7.

Bielski BH (1991) Studies of hypervalent iron. *Free Radic Res Commun* 12-13: 469-77.

Billups B, Rossi D, Attwell D (1996) Anion conductance behavior of the glutamate uptake carrier in salamander retinal glial cells. *J Neurosci* 16: 6722-31.

Blaschke M, Keller BU, Rivosecchi R, Hollmann M, Heinemann S, Konnerth A (1993) A single amino acid determines the subunit-specific spider toxin block of alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate/kainate receptor channels. *Proc Natl Acad Sci U S A* 90: 6528-32.

Bleakman D, Lodge D (1998) Neuropharmacology of AMPA and kainate receptors. *Neuropharm* 37: 1187-1204.

Blight AR, Decrescito V (1986) Morphometric analysis of experimental spinal cord injury in the cat: the relation of injury intensity to survival of myelinated axons. *Neuroscience* 19: 321-41.

Bochet P, Audinat E, Lambolez B, Crepel F, Rossier J, Iino M, Tsuzuki K, Ozawa S (1994) Subunit composition at the single-cell level explains functional properties of a glutamate-gated channel. *Neuron* 12: 383-8.

Bostock H, Grafe P (1985) Activity-dependent excitability changes in normal and demyelinated rat spinal root axons. *J Physiol (Lond)* 365: 239-257.

Bouvier M, Szatkowski M, Amato A, Attwell D (1992) The glial cell glutamate uptake carrier countertransports pH-changing anions. *Nature* 360: 471-4.

Bracken MB, Shepard MJ, Collins WF, Holford TR, Young W, Baskin DS, Eisenberg HM, Flamm E, Leo-Summers L, Maroon J, et al (1990) A randomized, controlled trial of methylprednisolone or naloxone in the treatment of acute spinal-cord injury. Results of the Second National Acute Spinal Cord Injury Study. *N Engl J Med* 322: 1405-11.

Bracken MB, Shepard MJ, Holford TR, Leo-Summers L, Aldrich EF, Fazl M, Fehlings M, Herr DL, Hitchon PW, Marshall LF, Nockels RP, Pascale V, Perot PL Jr, Piepmeier J, Sonntag VK, Wagner F, Wilberger JE, Winn HR, Young W (1997) Administration of methylprednisolone for 24 or 48 hours or tirilazad mesylate for 48 hours in the treatment of acute spinal cord injury. Results of the Third National Acute Spinal Cord Injury Randomized Controlled Trial. National Acute Spinal Cord Injury Study. *JAMA* 277: 1597-604.

Brorson JR, Bleakman D, Chard PS, Miller RJ (1992) Calcium directly permeates kainate/alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors in cultured cerebellar Purkinje neurons. *Mol Pharmacol* 41: 603-8.

Brorson JR, Manzillo PA, Miller RJ (1994) Ca^{2+} entry via AMPA/KA receptors and excitotoxicity in cultured cerebellar Purkinje cells. *J Neurosci* 14: 187-97.

Brorson JR, Zhang Z, Vandenberghe W (1999) Ca^{2+} permeation of AMPA receptors in cerebellar neurons expressing glu receptor 2. *J Neurosci* 19: 9149-59.

Buchan A, Li H, Pulsinelli WA (1991) The N-methyl-D-aspartate antagonist, MK-801, fails to protect against neuronal damage caused by transient, severe forebrain ischemia in adult rats. *J Neurosci* 11: 1049-56.

Buchan A, Pulsinelli WA (1990) Hypothermia but not the N-Methyl-D-Aspartate antagonist, MK-801, attenuates neuronal damage in gerbils subjected to transient global ischemia. *J Neurosci* 10: 311-6.

Buki A, Okonkwo DO, Povlishock JT (1999) Postinjury cyclosporin A administration limits axonal damage and disconnection in traumatic brain injury. *J Neurotrauma* 16: 511-21.

Buki A, Okonkwo DO, Wang KK, Povlishock JT (2000) Cytochrome c release and caspase activation in traumatic axonal injury. *J Neurosci* 20: 2825-34.

Bullock R, Fujisawa H (1992) The role of glutamate antagonists for the treatment of CNS injury. *J Neurotrauma* 9 (Suppl 2): S443-62.

Bullock R, Zauner A, Woodward JJ, Myseros J, Choi SC, Ward JD, Marmarou A, Young HF (1998) Factors affecting excitatory amino acid release following severe human head injury. *J Neurosurg* 89: 507-18.

Bunge RP, Puckett WR, Becerra JL, Marcillo A, Quencer RM (1993) Observations on the pathology of human spinal cord injury. A review and classification of 22 new cases with details from a case of chronic cord compression with extensive focal demyelination. *Adv Neurol* 59: 75-89.

Bunge RP, Puckett WR, Hiester ED (1997) Observations on the pathology of several types of human spinal cord injury, with emphasis on the astrocyte response to penetrating injuries. *Adv Neurol* 72: 305-15.

Burnashev N (1996) Calcium permeability of glutamate-gated channels in the central nervous system. *Curr Opin Neurobiol* 6: 311-7.

Burnashev N, Khodorova A, Jonas P, Helm PJ, Wisden W, Monyer H, Seeburg PH, Sakmann B (1992) Calcium-permeable AMPA-kainate receptors in fusiform cerebellar glial cells. *Science* 256: 1566-70.

Carlson NG, Gahring LC, Twyman RE, Rogers SW (1997) Identification of amino acids in the glutamate receptor, GluR3, important for antibody-binding and receptor-specific activation. *J Biol Chem* 272: 11295-301.

Carriedo SG, Yin HZ, Sensi SL, Weiss JH (1998) Rapid Ca^{2+} entry through Ca^{2+} -permeable AMPA/Kainate channels triggers marked intracellular Ca^{2+} rises and consequent oxygen radical production. *J Neurosci* 18: 7727-38.

Carriedo SG, Yin HZ, Weiss JH (1996) Motor neurons are selectively vulnerable to AMPA/kainate receptor-mediated injury in vitro. *J Neurosci* 16: 4069-79.

Chakraborti T, Das S, Mondal M, Roychoudhury S, Chakraborti S (1999) Oxidant, mitochondria and calcium: an overview. *Cell Signal* 11: 77-85.

Chaudhry FA, Lehre KP, van Lookeren Campagne M, Ottersen OP, Danbolt NC, Storm-Mathisen J (1995) Glutamate transporters in glial plasma membranes: highly differentiated localizations revealed by quantitative ultrastructural immunocytochemistry. *Neuron* 15: 711-20.

Chen GQ, Gouaux E (1997) Overexpression of a glutamate receptor (GluR2) ligand binding domain in *Escherichia coli*: application of a novel protein folding screen. *Proc Natl Acad Sci U S A* 94: 13431-6.

Chen JW, Zhang L, Lian X, Hwang F (1992) Effect of hydroxyl radical on Na⁺-K⁺-ATPase activity of the brain microsomal membranes. *Cell Biol Int Rep* 16: 927-36.

Chinopoulos C, Tretter L, Rozsa A, Adam-Vizi V (2000) Exacerbated responses to oxidative stress by an Na⁺ load in isolated nerve terminals: the role of ATP depletion and rise of [Ca(2+)](i). *J Neurosci* 20: 2094-103.

Chiu SY, Kriegler S (1994) Neurotransmitter-mediated signaling between axons and glial cells. *Glia* 11: 191-200.

Chiu SY, Ritchie JM (1982) Evidence for the presence of potassium channels in the internode of frog myelinated nerve fibres. *J Physiol (Lond)* 322: 485-501.

Choi DW (1992a) Comment: modeling for drug development in spinal cord injury. *J Neurotrauma* 9: 135-6.

Choi DW (1992b) Excitotoxic cell death. *J Neurobiol* 23: 1261-76.

Choi DW, Rothman SM (1990) The role of glutamate neurotoxicity in hypoxic-ischemic neuronal death. *Annu Rev Neurosci* 13: 171-82.

Clarke PG (1990) Developmental cell death: morphological diversity and multiple mechanisms. *Anat Embryol* 181: 195-213.

Clements JD, Feltz A, Sahara Y, Westbrook GL (1998) Activation kinetics of AMPA receptor channels reveal the number of functional agonist binding sites. *J Neurosci* 18: 119-27.

Collins WF (1983) A review and update of experiment and clinical studies of spinal cord injury. *Paraplegia* 21: 204-19.

Compton MM (1992) A biochemical hallmark of apoptosis: internucleosomal degradation of the genome. *Cancer Metastasis Rev* 11: 105-19.

Conradt M, Stoffel W (1995) Functional analysis of the high affinity, Na⁺-dependent glutamate transporter GLAST-1 by site-directed mutagenesis. *J Biol Chem* 270: 25207-12.

Cooper MS (1995) Intercellular signaling in neuronal-glial networks. *Biosystems* 34: 65-85.

Corley SM, Yong VW (1999) Oligodendrocyte death following CNS trauma. *Soc Neurosci Abs* 25: 739 (292.9).

Coyle JT, Puttfarcken P (1993) Oxidative stress, glutamate, and neurodegenerative disorders. *Science* 262: 689-95.

Croall DE, DeMartino GN (1991) Calcium-activated neutral protease (calpain) system: structure, function, and regulation. *Physiol Rev* 71: 813-47.

Crompton M, Andreeva L (1993) On the involvement of a mitochondrial pore in reperfusion injury. *Basic Res Cardiol* 88: 513-23.

Crowe MJ, Bresnahan JC, Shuman SL, Masters JN, Beattie MS (1997) Apoptosis and delayed degeneration after spinal cord injury in rats and monkeys. *Nat Med* 3: 73-6.

D'Ambrosio R, Maris DO, Grady MS, Winn HR, Janigro D (1999) Impaired K⁺ homeostasis and altered electrophysiological properties of post-traumatic hippocampal glia. *J Neurosci* 19: 8152-62.

Danbolt NC, Pines G, Kanner BI (1990) Purification and reconstitution of the sodium- and potassium-coupled glutamate transport glycoprotein from rat brain. *Biochemistry* 29: 6734-40.

Danbolt NC, Storm-Mathisen J, Kanner BI (1992) An [Na⁺ + K⁺]coupled L-glutamate transporter purified from rat brain is located in glial cell processes. *Neuroscience* 51: 295-310.

Dani JW, Chernjavsky A, Smith SJ (1992) Neuronal activity triggers calcium waves in hippocampal astrocyte networks. *Neuron* 8: 429-40.

Dani JW, Smith SJ (1995) The triggering of astrocytic calcium waves by NMDA-induced neuronal activation. *Ciba Found Symp* 188: 195-205.

David JC, Yamada KA, Bagwe MR, Goldberg MP (1996) AMPA receptor activation is rapidly toxic to cortical astrocytes when desensitization is blocked. *J Neurosci* 16: 200-9.

Dehnes Y, Chaudhry FA, Ullensvang K, Lehre KP, Storm-Mathisen J, Danbolt NC (1998) The glutamate transporter EAAT4 in rat cerebellar Purkinje cells: a glutamate-gated chloride channel concentrated near the synapse in parts of the dendritic membrane facing astroglia. *J Neurosci* 18: 3606-19.

Derouiche A, Rauen T (1995) Coincidence of L-glutamate/L-aspartate transporter (GLAST) and glutamine synthetase (GS) immunoreactions in retinal glia: evidence for coupling of GLAST and GS in transmitter clearance. *J Neurosci Res* 42: 131-43.

Dingledine R, Hume RI, Heinemann SF (1992) Structural determinants of barium permeation and rectification in non- NMDA glutamate receptor channels. *J Neurosci* 12: 4080-7.

Dirnagl U, Iadecola C, Moskowitz MA (1999) Pathobiology of ischaemic stroke: an integrated view. *Trends Neurosci* 22: 391-7.

Domercq M, Matute C (1999) Expression of glutamate transporters in the adult bovine corpus callosum. *Brain Res Mol Brain Res* 67: 296-302.

Domercq M, Sanchez-Gomez MV, Areso P, Matute C (1999) Expression of glutamate transporters in rat optic nerve oligodendrocytes. *Eur J Neurosci* 11: 2226-36.

Donevan SD, Rogawski MA (1993) GYKI 52466, a 2,3-benzodiazepine, is a highly selective, noncompetitive antagonist of AMPA/kainate receptor responses. *Neuron* 10: 51-9.

Donevan SD, Rogawski MA (1995) Intracellular polyamines mediate inward rectification of Ca^{2+} - permeable alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors. *Proc Natl Acad Sci U S A* 92: 9298-302.

Drejer J, Benveniste H, Diemer NH, Schousboe A (1985) Cellular origin of ischemia-induced glutamate release from brain tissue in vivo and in vitro. *J Neurochem* 45: 145-51.

Drejer J, Larsson OM, Schousboe A (1983) Characterization of uptake and release processes for D- and L-aspartate in primary cultures of astrocytes and cerebellar granule cells. *Neurochem Res* 8: 231-43.

Du C, Hu R, Csernansky CA, Hsu CY, Choi DW (1996) Very delayed infarction after mild focal cerebral ischemia: a role for apoptosis? J Cereb Blood Flow Metab 16: 195-201.

Duffy S , MacVicar BA (1996) In vitro ischemia promotes calcium influx and intracellular calcium release in hippocampal astrocytes. J Neurosci 16: 71-81.

Dykens JA (1994) Isolated cerebral and cerebellar mitochondria produce free radicals when exposed to elevated Ca^{2+} and Na^{+} : implications for neurodegeneration. J Neurochem 63: 584-91.

Euler T, Wassle H (1995) Immunocytochemical identification of cone bipolar cells in the rat retina. J Comp Neurol 361: 461-78.

Evans PD, Reale V, Merzon RM, Villegas J (1991) Mechanisms of axon-Schwann cell signaling in the squid nerve fiber. Ann N Y Acad Sci 633: 434-47.

Faden AI, Ivanova SA, Yakovlev AG, Mukhin AG (1997) Neuroprotective effects of group III mGluR in traumatic neuronal injury. J Neurotrauma 14: 885-95.

Fagni L, Chavis P, Ango F, Bockaert J (2000) Complex interactions between mGluRs, intracellular Ca^{2+} stores and ion channels in neurons. Trends Neurosci 23: 80-8.

Fairman WA, Vandenberg RJ, Arriza JL, Kavanaugh MP, Amara SG (1995) An excitatory amino-acid transporter with properties of a ligand-gated chloride channel. *Nature* 375: 599-603.

Farooque M, Olsson Y, Hillered L (1997) Pretreatment with alpha-phenyl-N-tert-butyl-nitron (PBN) improves energy metabolism after spinal cord injury in rats. *J Neurotrauma* 14: 469-76.

Fehlings MG, Tator CH (1995) The relationships among the severity of spinal cord injury, residual neurological function, axon counts, and counts of retrogradely labeled neurons after experimental spinal cord injury. *Exp Neurol* 132: 220-228.

Fern R, Moller T (2000) Rapid ischemic cell death in immature oligodendrocytes: A fatal glutamate release feedback loop. *J Neurosci* 20: 34-42.

Fern R, Ransom BR, Waxman SG (1995) Voltage-gated calcium channels in CNS white matter: role in anoxic injury. *J Neurophysiol* 74: 369-377.

Finkbeiner SM (1993) Glial calcium. *Glia* 9: 83-104.

Follis F, Scremin OU, Blisard KS, Scremin AM, Pett SB, Scott WJ, Kessler RM, Wernly JA (1993) Selective vulnerability of white matter during spinal cord ischemia. *J Cereb Blood Flow Metab* 13: 170-8.

Francel PC, Long BA, Malik JM, Tribble C, Jane JA, Kron IL (1993) Limiting ischemic spinal cord injury using a free radical scavenger 21- aminosteroid and/or cerebrospinal fluid drainage. *J Neurosurg* 79: 742-51.

Funk GD, Smith JC, Feldman JL (1995) Modulation of neural network activity in vitro by cyclothiazide, a drug that blocks desensitization of AMPA receptors. *J Neurosci* 15: 4046-56.

Furuichi T, Furutama D, Hakamata Y, Nakai J, Takeshima H, Mikoshiba K (1994) Multiple types of ryanodine receptor/ Ca^{2+} release channels are differentially expressed in rabbit brain. *J Neurosci* 14: 4794-805.

Gallo V, Patrizio M, Levi G (1991) GABA release triggered by the activation of neuron-like non-NMDA receptors in cultured type 2 astrocytes is carrier-mediated. *Glia* 4: 245-55.

Gallo V, Russell JT (1995) Excitatory amino acid receptors in glia: different subtypes for distinct functions? *J Neurosci Res* 42: 1-8.

Garcia-Barcina JM, Matute C (1996) Expression of kainate-selective glutamate receptor subunits in glial cells of the adult bovine white matter. *Eur J Neurosci* 8: 2379-2387.

Garcia-Barcina JM, Matute C (1998) AMPA-selective glutamate receptor subunits in glial cells of the adult bovine white matter. *Brain Res Mol Brain Res* 53: 270-6.

Garthwaite G, Brown G, Batchelor AM, Goodwin DA, Garthwaite J (1999) Mechanisms of ischaemic damage to central white matter axons: a quantitative histological analysis using rat optic nerve. *Neuroscience* 94: 1219-30.

Gegelashvili G, Schousboe A (1997) High affinity glutamate transporters: regulation of expression and activity. *Mol Pharmacol* 52: 6-15.

Gegelashvili G, Schousboe A (1998) Cellular distribution and kinetic properties of high-affinity glutamate transporters. *Brain Res Bull* 45: 233-8.

Geiger JR, Melcher T, Koh DS, Sakmann B, Seeburg PH, Jonas P, Monyer H (1995) Relative abundance of subunit mRNAs determines gating and Ca^{2+} permeability of AMPA receptors in principal neurons and interneurons in rat CNS. *Neuron* 15: 193-204.

Geisler FH, Dorsey FC, Coleman WP (1991) Recovery of motor function after spinal-cord injury--a randomized, placebo-controlled trial with GM-1 ganglioside. *N Engl J Med* 324: 1829-38.

Gemba T, Oshima T, Ninomiya M (1994) Glutamate efflux via the reversal of the sodium-dependent glutamate transporter caused by glycolytic inhibition in rat cultured astrocytes. *Neurosci* 63: 789-95.

Goldberg MP, Choi DW (1993) Combined oxygen and glucose deprivation in cortical cell culture: calcium-dependent and calcium-independent mechanisms of neuronal injury. *J Neurosci* 13: 3510-24.

Goldman WF, Yarowsky PJ, Juhaszova M, Krueger BK, Blaustein MP (1994) Sodium/calcium exchange in rat cortical astrocytes. *J Neurosci* 14: 5834-43.

Goldstein PA, Lee CJ, MacDermott AB (1995) Variable distributions of Ca^{2+} -permeable and Ca^{2+} -impermeable AMPA receptors on embryonic rat dorsal horn neurons. *J Neurophysiol* 73: 2522-34.

Gong QZ, Delahunty TM, Hamm RJ, Lyeth BG (1995) Metabotropic glutamate antagonist, MCPG, treatment of traumatic brain injury in rats. *Brain Res* 700: 299-302.

Gorter JA, Petrozzino JJ, Aronica EM, Rosenbaum DM, Opitz T, Bennett MV, Connor JA, Zukin RS (1997) Global ischemia induces downregulation of Glur2 mRNA and increases AMPA receptor-mediated Ca^{2+} influx in hippocampal CA1 neurons of gerbil. *J Neurosci* 17: 6179-88.

Green DR , Reed JC (1998) Mitochondria and apoptosis. Science 281: 1309-12.

Grosche J, Matyash V, Moller T, Werkhratsky A, Reichenbach A, Kettenmann H (1999) Microdomains for neuron-glia interaction: parallel fiber signaling to Bergmann glial cells. Nat Neurosci 2: 139-43.

Grunert U, Martin PR, Wassle H (1994) Immunocytochemical analysis of bipolar cells in the macaque monkey retina. J Comp Neurol 348: 607-27.

Gu JG, Albuquerque C, Lee CJ, MacDermott AB (1996) Synaptic strengthening through activation of Ca^{2+} -permeable AMPA receptors. Nature 381: 793-6.

Gwag BJ, Lobner D, Koh JY, Wie MB, Choi DW (1995) Blockade of glutamate receptors unmasks neuronal apoptosis after oxygen- glucose deprivation in vitro. Neuroscience 68: 615-9.

Hall ED (1989) Free radicals and CNS injury. Crit Care Clin 5: 793-805.

Hall ED (1992) The neuroprotective pharmacology of methylprednisolone. J Neurosurg 76: 13-22.

Hall ED (1996) Lipid peroxidation. Adv Neurol 71: 247-257.

Hall ED, Braugher JM, McCall JM (1992) Antioxidant effects in brain and spinal cord injury. *J Neurotrauma* 9 (Suppl 1): S165-72.

Halliwell B , Gutteridge JM (1984) Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochem J* 219: 1-14.

Herlitze S, Raditsch M, Ruppersberg JP, Jahn W, Monyer H, Schoepfer R, Witzemann V (1993) Argiotoxin detects molecular differences in AMPA receptor channels. *Neuron* 10: 1131-40.

Heron A, Pollard H, Dessi F, Moreau J, Lasbennes F, Ben-Ari Y, Charriaut-Marlangue C (1993) Regional variability in DNA fragmentation after global ischemia evidenced by combined histological and gel electrophoresis observations in the rat brain. *J Neurochem* 61: 1973-6.

Hertz L, Peng L (1992) Energy metabolism at the cellular level of the CNS. *Can J Physiol Pharmacol* 70: S145-S157.

Higuchi M, Single FN, Kohler M, Sommer B, Sprengel R, Seeburg PH (1993) RNA editing of AMPA receptor subunit GluR-B: a base-paired intron-exon structure determines position and efficiency. *Cell* 75: 1361-70.

Holgado A, Beauge L (1995) The Na^+ - Ca^{2+} exchange system in rat glial cells in culture: activation by external monovalent cations. *Glia* 14: 77-86.

Holgado A, Beauge L (1996) Effects of external monovalent cations on Na^+ - Ca^{2+} exchange in cultured rat glial cells. *Ann N Y Acad Sci* 779: 279-281.

Hollmann M, Hartley M, Heinemann S (1991) Ca^{2+} permeability of KA-AMPA--gated glutamate receptor channels depends on subunit composition. *Science* 252: 851-3.

Hollmann M, Heinemann S (1994) Cloned glutamate receptors. *Annu Rev Neurosci* 17: 31-108.

Hosli E, Hosli L (1993) Receptors for neurotransmitters on astrocytes in the mammalian central nervous system. *Prog Neurobiol* 40: 477-506.

Hoyt KR, Arden SR, Aizenman E, Reynolds IJ (1998) Reverse Na^+ / Ca^{2+} exchange contributes to glutamate-induced intracellular Ca^{2+} concentration increases in cultured rat forebrain neurons. *Mol Pharmacol* 53: 742-9.

Huang WH, Wang Y, Askari A (1992) $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$: inactivation and degradation induced by oxygen radicals. *Int J Biochem* 24: 621-6.

Husain J, Juurlink BH (1995) Oligodendroglial precursor cell susceptibility to hypoxia is related to poor ability to cope with reactive oxygen species. *Brain Res.* 698: 86-94.

Iino M, Koike M, Isa T, Ozawa S (1996) Voltage-dependent blockage of Ca^{2+} -permeable AMPA receptors by joro spider toxin in cultured rat hippocampal neurones. *J Physiol (Lond)* 496: 431-7.

Iino M, Ozawa S, Tsuzuki K (1990) Permeation of calcium through excitatory amino acid receptor channels in cultured rat hippocampal neurones. *J Physiol (Lond)* 424: 151-65.

Ikeda Y, Long DM (1990) The molecular basis of brain injury and brain edema: the role of oxygen free radicals. *Neurosurgery* 27: 1-11.

Imaizumi T, Kocsis JD, Waxman SG (1997) Anoxic injury in the rat spinal cord - pharmacological evidence for multiple steps in Ca^{2+} -dependent injury of the dorsal columns. *J Neurotrauma* 14: 299-311.

Imaizumi T, Kocsis JD, Waxman SG (1999) The role of voltage-gated Ca^{2+} channels in anoxic injury of spinal cord white matter. *Brain Res.* 817: 84-92.

Isa T, Itazawa S, Iino M, Tsuzuki K, Ozawa S (1996) Distribution of neurones expressing inwardly rectifying and Ca^{2+} - permeable AMPA receptors in rat hippocampal slices. *J Physiol (Lond)* 491: 719-33.

Itazawa SI, Isa T, Ozawa S (1997) Inwardly rectifying and Ca^{2+} -permeable AMPA-type glutamate receptor channels in rat neocortical neurons. *J Neurophysiol* 78: 2592-601.

Itoh T, Itoh A, Horiuchi K, Pleasure D (1998) AMPA receptor-mediated excitotoxicity in human NT2-N neurons results from loss of intracellular Ca^{2+} homeostasis following marked elevation of intracellular Na^{+} . *J Neurochem* 71: 112-24.

Jacobson MD (1996) Reactive oxygen species and programmed cell death. *Trends Biochem Sci* 21: 83-6.

Jacobson MD (1997) Apoptosis: Bcl-2-related proteins get connected. *Curr Biol* 7: R277-81.

Jahromi BS, Robitaille R, Charlton MP (1992) Transmitter release increases intracellular calcium in perisynaptic Schwann cells in situ. *Neuron* 8: 1069-77.

Jelinski SE, Yager JY, Juurlink BH (1999) Preferential injury of oligodendroblasts by a short hypoxic-ischemic insult. *Brain Res* 815: 150-3.

Jensen AM, Chiu SY (1993) Expression of glutamate receptor genes in white matter: developing and adult rat optic nerve. *J Neurosci* 13: 1664-75.

Jia Z, Agopyan N, Miu P, Xiong Z, Henderson J, Gerlai R, Taverna FA, Velumian A, MacDonald J, Carlen P, Abramow-Newerly W, Roder J (1996) Enhanced LTP in mice deficient in the AMPA receptor GluR2. *Neuron* 17: 945-56.

Jiang Q, Stys PK (2000) Calpain inhibitors confer biochemical, but not electrophysiological, protection against anoxia in rat optic nerves. *J Neurochem* 74: 2101-7.

Jonas P, Burnashev N (1995) Molecular mechanisms controlling calcium entry through AMPA-type glutamate receptor channels. *Neuron* 15: 987-90.

Jonas P, Racca C, Sakmann B, Seeburg PH, Monyer H (1994) Differences in Ca^{2+} permeability of AMPA-type glutamate receptor channels in neocortical neurons caused by differential GluR-B subunit expression. *Neuron* 12: 1281-9.

Kakulas BA, Lorimer RL, Gubbay AD (1998) White matter changes in human spinal cord injury. In: *Spinal cord monitoring* (E. Stalberg, H. S. Sharma and Y. Olsson ed), pp 395-408. New York: Springer-Verlag.

Kanai Y, Hediger MA (1992) Primary structure and functional characterization of a high-affinity glutamate transporter. *Nature* 360: 467-71.

Kanai Y, Stelzner M, Nussberger S, Khawaja S, Hebert SC, Smith CP, Hediger MA (1994) The neuronal and epithelial human high affinity glutamate transporter. Insights into structure and mechanism of transport. *J Biol Chem* 269: 20599-606.

Kanner BI, Sharon I (1978) Solubilization and reconstitution of the L-glutamic acid transporter from rat brain. *FEBS Lett* 94: 245-8.

Kask K, Zamanillo D, Rozov A, Burnashev N, Sprengel R, Seeburg PH (1998) The AMPA receptor subunit GluR-B in its Q/R site-unedited form is not essential for brain development and function. *Proc Natl Acad Sci U S A* 95: 13777-82.

Katayama Y, Kawamata T, Tamura T, Hovda DA, Becker DP, Tsubokawa T (1991) Calcium-dependent glutamate release concomitant with massive potassium flux during cerebral ischemia in vivo. *Brain Res* 558: 136-40.

Khodakhah K, Ogden D (1993) Functional heterogeneity of calcium release by inositol trisphosphate in single Purkinje neurones, cultured cerebellar astrocytes, and peripheral tissues. *Proc Natl Acad Sci U S A* 90: 4976-80.

Kimelberg HK, Goderie SK, Higman S, Pang S, Waniewski RA (1990) Swelling-induced release of glutamate, aspartate, and taurine from astrocyte cultures. *J Neurosci* 10: 1583-91.

Kinuta Y, Kimura M, Itokawa Y, Ishikawa M, Kikuchi H (1989) Changes in xanthine oxidase in ischemic rat brain. *J Neurosurg* 71: 417-420.

Kirischuk S, Kettenmann H, Verkhratsky A (1997) $\text{Na}^+/\text{Ca}^{2+}$ exchanger modulates kainate-triggered Ca^{2+} signaling in Bergmann glial cells in situ. *FASEB J* 11: 566-72.

Koh DS, Burnashev N, Jonas P (1995) Block of native Ca^{2+} -permeable AMPA receptors in rat brain by intracellular polyamines generates double rectification. *J Physiol (Lond)* 486: 305-12.

Kondo K, Hashimoto H, Kitanaka J, Sawada M, Suzumura A, Marunouchi T, Baba A (1995) Expression of glutamate transporters in cultured glial cells. *Neurosci Lett* 188: 140-2.

Kondo M, Sumino R, Okado H (1997) Combinations of AMPA receptor subunit expression in individual cortical neurons correlate with expression of specific calcium-binding proteins. *J Neurosci* 17: 1570-81.

Kriegler S, Chiu SY (1993) Calcium signaling of glial cells along mammalian axons. *J Neurosci* 13: 4229-4245.

Kristian T, Siesjo BK (1998) Calcium in ischemic cell death. *Stroke* 29: 705-718.

Kroemer G, Dallaporta B, Resche-Rigon M (1998) The mitochondrial death/life regulator in apoptosis and necrosis. *Annu Rev Physiol* 60: 619-42.

Kroemer G, Reed JC (2000) Mitochondrial control of cell death. *Nat Med* 6: 513-9.

Kugler P, Schmitt A (1999) Glutamate transporter EAAC1 is expressed in neurons and glial cells in the rat nervous system. *Glia* 27: 129-142.

Kwo S, Young W, Decrescito V (1989) Spinal cord sodium, potassium, calcium, and water concentration changes in rats after graded contusion injury. *J Neurotrauma* 6: 13-24.

Le Peillet E, Arvin B, Moncada C, Meldrum BS (1992) The non-NMDA antagonists, NBQX and GYKI 52466, protect against cortical and striatal cell loss following transient global ischaemia in the rat. *Brain Res* 571: 115-20.

Lee JM, Zipfel GJ, Choi DW (1999) The changing landscape of ischaemic brain injury mechanisms. *Nature* 399: A7-14.

Lehning EJ, Doshi R, Isaksson N, Stys PK, Lopachin RM (1996) Mechanisms of injury-induced calcium entry into peripheral nerve myelinated axons: role of reverse sodium-calcium exchange. *J Neurochem* 66: 493-500.

Lehre KP, Levy LM, Ottersen OP, Storm-Mathisen J, Danbolt NC (1995) Differential expression of two glial glutamate transporters in the rat brain: quantitative and immunocytochemical observations. *J Neurosci* 15: 1835-53.

Leppanen LL, Stys PK (1997) Ion transport and membrane potential in CNS myelinated axons. II: effects of metabolic inhibition. *J Neurophysiol* 78: 2095-2107.

Lerma J, Morales M, Ibarz JM, Somohano F (1994) Rectification properties and Ca^{2+} permeability of glutamate receptor channels in hippocampal cells. *Eur J Neurosci* 6: 1080-8.

Lev-Ram V, Ellisman MH (1995) Axonal activation-induced calcium transients in myelinating Schwann cells, sources, and mechanisms. *J Neurosci* 15: 2628-37.

Levy LM, Lehre KP, Rolstad B, Danbolt NC (1993) A monoclonal antibody raised against an $[\text{Na}^{+}+\text{K}^{+}]$ -coupled L-glutamate transporter purified from rat brain confirms glial cell localization. *FEBS Lett* 317: 79-84.

Li GL, Farooque M, Holtz A, Olsson Y (1999) Apoptosis of oligodendrocytes occurs for long distances away from the primary injury after compression trauma to rat spinal cord. *Acta Neuropathol (Berl)* 98: 473-80.

Li HS, Niedzielski AS, Beisel KW, Hiel H, Wenthold RJ, Morley BJ (1994) Identification of a glutamate/aspartate transporter in the rat cochlea. *Hear Res* 78: 235-42.

Li S, Jiang Q, Stys PK (2000) Important role of reverse Na^+ - Ca^{2+} exchange in spinal cord white matter injury at physiological temperature. *J Neurophysiol* 84: 1116-9.

Li S, Tator CH (1998) Spinal cord blood flow and evoked potentials as outcome measures for experimental spinal cord injury. In: *Spinal cord monitoring* (E. Stalberg, H. S. Sharma and Y. Olsson ed), pp 365-392. Springer-Verlag.

Liaw KY, Wei TC, Hsu SC, Lin JK (1985) Effect of severe injury and critical illness on high-energy phosphates in human liver and muscle. *J Trauma* 25: 628-33.

Lieberman EM (1991) Role of glutamate in axon-Schwann cell signaling in the squid. *Ann N Y Acad Sci* 633: 448-57.

Lieberman EM, Sanzenbacher E (1992) Mechanisms of glutamate activation of axon-to-Schwann cell signaling in the squid. *Neuroscience* 47: 931-9.

Linnik MD, Zobrist RH, Hatfield MD (1993) Evidence supporting a role for programmed cell death in focal cerebral ischemia in rats. *Stroke* 24: 2002-8.

Lipton P (1999) Ischemic cell death in brain neurons. *Physiol Rev* 79: 1431-568.

Liu D, Liu J, Wen J (1999a) Elevation of hydrogen peroxide after spinal cord injury detected by using the Fenton reaction. *Free Radic Biol Med* 27: 478-82.

Liu D, Sybert TE, Qian H, Liu J (1998) Superoxide production after spinal injury detected by microperfusion of cytochrome c. *Free Radic Biol Med* 25: 298-304.

Liu D, Wen J, Liu J, Li L (1999b) The roles of free radicals in amyotrophic lateral sclerosis: reactive oxygen species and elevated oxidation of protein, DNA, and membrane phospholipids. *Faseb J* 13: 2318-28.

Liu XZ, Xu XM, Hu R, Du C, Zhang SX, McDonald JW, Dong HX, Wu YJ, Fan GS, Jacquin MF, Hsu CY, Choi DW (1997) Neuronal and glial apoptosis after traumatic spinal cord injury. *J Neurosci* 17: 5395-406.

Lomeli H, Mosbacher J, Melcher T, Hoyer T, Geiger JR, Kuner T, Monyer H, Higuchi M, Bach A, Seeburg PH (1994) Control of kinetic properties of AMPA receptor channels by nuclear RNA editing. *Science* 266: 1709-13.

Longuemare MC, Swanson RA (1997) Net glutamate release from astrocytes is not induced by extracellular potassium concentrations attainable in brain. *J Neurochem* 69: 879-82.

Lopachin RM (1999) Intraneuronal ion distribution during experimental oxygen/glucose deprivation. Routes of ion flux as targets of neuroprotective strategies. *Ann N Y Acad Sci* 890: 191-203.

LoPachin RM, Gaughan CL, Lehning EJ, Kaneko Y, Kelly TM, Blight A (1999) Experimental spinal cord injury: spatiotemporal characterization of elemental concentrations and water contents in axons and neuroglia. *J Neurophysiol* 82: 2143-53.

LoPachin RM, Stys PK (1995) Elemental composition and water content of rat optic nerve myelinated axons and glial cells: effects of in vitro anoxia and reoxygenation. *J Neurosci* 15: 6735-6746.

Louis JC, Magal E, Takayama S, Varon S (1993) CNTF protection of oligodendrocytes against natural and tumor necrosis factor-induced death. *Science* 259: 689-92.

Lucius R, Sievers J (1996) Postnatal retinal ganglion cells in vitro: protection against reactive oxygen species (ROS)-induced axonal degeneration by cocultured astrocytes. *Brain Res* 743: 56-62.

Mack KJ, Kriegler S, Chang S, Chiu SY (1994) Transcription factor expression is induced by axonal stimulation and glutamate in the glia of the developing optic nerve. *Brain Res Mol Brain Res* 23: 73-80.

MacManus JP, Rasquinha I, Black MA, Laferriere NB, Monette R, Walker T, Morley P (1997) Glutamate-treated rat cortical neuronal cultures die in a way different from the classical apoptosis induced by staurosporine. *Exp Cell Res* 233: 310-20.

Magazanik LG, Buldakova SL, Samoilova MV, Gmiro VE, Mellor IR, Usherwood PN (1997) Block of open channels of recombinant AMPA receptors and native AMPA/kainate receptors by adamantane derivatives. *J Physiol (Lond)* 505: 655-63.

Maire JC, Medilanski J, Straub RW (1982) Uptake of adenosine and release of adenine derivatives in mammalian non- myelinated nerve fibres at rest and during activity. *J Physiol (Lond)* 323: 589-602.

Maire JC, Medilanski J, Straub RW (1984) Release of adenosine, inosine and hypoxanthine from rabbit non- myelinated nerve fibres at rest and during activity. *J Physiol (Lond)* 357: 67-77.

Manfras BJ, Rudert WA, Trucco M, Boehm BO (1994) Cloning and characterization of a glutamate transporter cDNA from human brain and pancreas. *Biochim Biophys Acta* 1195: 185-8.

Matute C, Sanchez-Gomez MV, Martinez-Millan L, Miledi R (1997) Glutamate receptor-mediated toxicity in optic nerve oligodendrocytes. *Proc Natl Acad Sci U S A* 94: 8830-8835.

McDonald JW, Althomsons SP, Hyrc KL, Choi DW, Goldberg MP (1998) Oligodendrocytes from forebrain are highly vulnerable to AMPA/kainate receptor-mediated excitotoxicity. *Nat Med* 4: 291-297.

McHale MK, Hall GF, Cohen MJ (1995) Early cytoskeletal changes following injury of giant spinal axons in the lamprey. *J Comp Neurol* 353: 25-37.

McMahon HT, Nicholls DG (1991) Transmitter glutamate release from isolated nerve terminals: evidence for biphasic release and triggering by localized Ca^{2+} . *J Neurochem* 56: 86-94.

Meissner G (1994) Ryanodine receptor/ Ca^{2+} release channels and their regulation by endogenous effectors. *Annu Rev Physiol* 56: 485-508.

Meldrum BS (1994) The role of glutamate in epilepsy and other CNS disorders. *Neurology* 44(11 Suppl 8): S14-23.

Meldrum BS, Swan JH, Leach MJ, Millan MH (1992) Reduction of glutamate release and protection against ischemic brain damage by BW 1003C87. *Brain Research* 593: 1-6.

Metzger F, Kulik A, Sendtner M, Ballanyi K (2000) Contribution of Ca^{2+} -permeable AMPA/KA receptors to glutamate-induced Ca^{2+} rise in embryonic lumbar motoneurons in situ. *J Neurophysiol* 83: 50-9.

Michaelis EK (1998) Molecular biology of glutamate receptors in the central nervous system and their role in excitotoxicity, oxidative stress and aging. *Prog Neurobiol* 54: 369-415.

Miyazaki I, Iwata-Ichikawa E, Asanuma M, Iida M, Ogawa N (1999) Bifemelane hydrochloride protects against cytotoxicity of hydrogen peroxide on cultured rat neuroblastoma cell line. *Neurochem Res* 24: 857-60.

Morioka M, Fukunaga K, Yasugawa S, Nagahiro S, Ushio Y, Miyamoto E (1992) Regional and temporal alterations in Ca^{2+} /calmodulin-dependent protein kinase II and calcineurin in the hippocampus of rat brain after transient forebrain ischemia. *J Neurochem* 58: 1798-809.

Morikawa E, Mori H, Kiyama Y, Mishina M, Asano T, Kirino T (1998). Attenuation of focal ischemic brain injury in mice deficient in the epsilon1 (NR2A) subunit of NMDA receptor. *J Neurosci* 18: 9727-32.

Mosbacher J, Schoepfer R, Monyer H, Burnashev N, Seeburg PH, Ruppersberg JP (1994) A molecular determinant for submillisecond desensitization in glutamate receptors. *Science* 266: 1059-62.

Nakayama T, Kawakami H, Tanaka K, Nakamura S (1996) Expression of three glutamate transporter subtype mRNAs in human brain regions and peripheral tissues. *Brain Res Mol Brain Res* 36: 189-92.

Nelson PJ, Dean GE, Aronson PS, Rudnick G (1983) Hydrogen ion cotransport by the renal brush border glutamate transporter. *Biochemistry* 22: 5459-63.

Nicholls DG, Budd SL (1998) Neuronal excitotoxicity: the role of mitochondria. *Biofactors* 8: 287-99.

Nicholls DG, Budd SL (2000) Mitochondria and neuronal survival. *Physiol Rev* 80: 315-60.

Nicholls DG, Ward MW (2000) Mitochondrial membrane potential and neuronal glutamate excitotoxicity: mortality and millivolts. *Trends Neurosci* 23: 166-74.

Nicoletti F, Bruno V, Copani A, Casabona G, Knopfel T (1996) Metabotropic glutamate receptors: a new target for the therapy of neurodegenerative disorders? Trends Neurosci 19: 267-71.

Nicoll RA, Malenka RC (1995) Contrasting properties of two forms of long-term potentiation in the hippocampus. Nature 377: 115-8.

Nicotera P, Bellomo G, Orrenius S (1992) Calcium-mediated mechanisms in chemically induced cell death. Annu Rev Pharmacol Toxicol 32: 449-70.

Nitatori T, Sato N, Waguri S, Karasawa Y, Araki H, Shibani K, Kominami E, Uchiyama Y (1995) Delayed neuronal death in the CA1 pyramidal cell layer of the gerbil hippocampus following transient ischemia is apoptosis. J Neurosci 15: 1001-11.

Noble LJ, Wrathall JR (1989) Correlative analyses of lesion development and functional status after graded spinal cord contusive injuries in the rat. Exp Neurol 103: 34-40.

Obrenovitch TP (1996) Origins of glutamate release in ischaemia. Acta Neurochir Suppl 66: 50-5.

Oguro K, Oguro N, Kojima T, Grooms SY, Calderone A, Zheng X, Bennett MV, Zukin RS (1999) Knockdown of AMPA receptor GluR2 expression causes delayed

neurodegeneration and increases damage by sublethal ischemia in hippocampal CA1 and CA3 neurons. *J Neurosci* 19: 9218-27.

Okonkwo DO, Buki A, Siman R, Povlishock JT (1999) Cyclosporin A limits calcium-induced axonal damage following traumatic brain injury. *Neuroreport* 10: 353-8.

Okonkwo DO, Povlishock JT (1999) An intrathecal bolus of cyclosporin A before injury preserves mitochondrial integrity and attenuates axonal disruption in traumatic brain injury. *J Cereb Blood Flow Metab* 19: 443-51.

Olney JW, Sharpe LG (1969) Brain lesions in an infant rhesus monkey treated with monosodium glutamate. *Science* 166: 386-8.

Orrenius S, McConkey DJ, Bellomo G, Nicotera P (1989) Role of Ca^{2+} in toxic cell killing. *Trends Pharmacol Sci* 10: 281-5.

Otis TS, Raman IM, Trussell LO (1995) AMPA receptors with high Ca^{2+} permeability mediate synaptic transmission in the avian auditory pathway. *J Physiol (Lond)* 482: 309-15.

Otis TS, Wu YC, Trussell LO (1996) Delayed clearance of transmitter and the role of glutamate transporters at synapses with multiple release sites. *J Neurosci* 16: 1634-44.

Ozawa S, Iino M (1993) Two distinct types of AMPA responses in cultured rat hippocampal neurons. *Neurosci Lett* 155: 187-90.

Ozawa S, Kamiya H, Tsuzuki K (1998) Glutamate receptors in the mammalian central nervous system. *Prog Neurobiol* 54: 581-618.

Pantoni L, Garcia JH, Gutierrez JA (1996) Cerebral white matter is highly vulnerable to ischemia. *Stroke* 27: 1641-1646.

Partin KM, Bowie D, Mayer ML (1995) Structural determinants of allosteric regulation in alternatively spliced AMPA receptors. *Neuron* 14: 833-43.

Partin KM, Fleck MW, Mayer ML (1996) AMPA receptor flip/flop mutants affecting deactivation, desensitization, and modulation by cyclothiazide, aniracetam, and thiocyanate. *J Neurosci* 16: 6634-47.

Pasti L, Pozzan T, Carmignoto G (1995) Long-lasting changes of calcium oscillations in astrocytes. A new form of glutamate-mediated plasticity. *J Biol Chem* 270: 15203-10.

Pasti L, Volterra A, Pozzan T, Carmignoto G (1997) Intracellular calcium oscillations in astrocytes: a highly plastic, bidirectional form of communication between neurons and astrocytes in situ. *J Neurosci* 17: 7817-30.

Patneau DK, Wright PW, Winters C, Mayer ML, Gallo V (1994) Glial cells of the oligodendrocyte lineage express both kainate- and AMPA-preferring subtypes of glutamate receptor. *Neuron* 12: 357-71.

Patt A, Harken AH, Burton LK, Rodell TC, Piermattei D, Schorr WJ, Parker NB, Berger EM, Horesh IR, Terada LS , al. e (1988) Xanthine oxidase-derived hydrogen peroxide contributes to ischemia reperfusion-induced edema in gerbil brains. *J Clin Invest* 81: 1556-1562.

Pellegrini-Giampietro DE, Gorter JA, Bennett MV, Zukin RS (1997) The GluR2 (GluR-B) hypothesis: Ca^{2+} -permeable AMPA receptors in neurological disorders. *Trends Neurosci* 20: 464-70.

Pellegrini-Giampietro DE, Pulsinelli WA, Zukin RS (1994) NMDA and non-NMDA receptor gene expression following global brain ischemia in rats: effect of NMDA and non-NMDA receptor antagonists. *J Neurochem* 62: 1067-73.

Pellegrini-Giampietro DE, Zukin RS, Bennett MV, Cho S, Pulsinelli WA (1992) Switch in glutamate receptor subunit gene expression in CA1 subfield of hippocampus following global ischemia in rats. *Proc Natl Acad Sci U S A* 89: 10499-10503.

Pende M, Holtzclaw LA, Curtis JL, Russell JT, Gallo V (1994) Glutamate regulates intracellular calcium and gene expression in oligodendrocyte progenitors through the

activation of DL-alpha-amino-3- hydroxy-5-methyl-4-isoxazolepropionic acid receptors.
Proc Natl Acad Sci U S A 91: 3215-9.

Perez Velazquez JL, Frantseva MV, Carlen PL (1997) In vitro ischemia promotes glutamate-mediated free radical generation and intracellular calcium accumulation in hippocampal pyramidal neurons. J Neurosci 17: 9085-94.

Pines G, Danbolt NC, Bjoras M, Zhang Y, Bendahan A, Eide L, Koepsell H, Storm-Mathisen J, Seeberg E, Kanner BI (1992) Cloning and expression of a rat brain L-glutamate transporter . Nature 360: 464-7.

Porter JT, McCarthy KD (1996) Hippocampal astrocytes in situ respond to glutamate released from synaptic terminals. J Neurosci 16: 5073-81.

Portera-Cailliau C, Hedreen JC, Price DL, Koliatsos VE (1995) Evidence for apoptotic cell death in Huntington disease and excitotoxic animal models. J Neurosci 15: 3775-87.

Povlishock JT, Kontos HA (1992) The role of oxygen radicals in the pathobiology of traumatic brain injury. Hum Cell 5: 345-53.

Quandt FN, MacVicar BA (1986) Calcium activated potassium channels in cultured astrocytes. Neuroscience 19: 29-41.

Raff MC, Barres BA, Burne JF, Coles HS, Ishizaki Y, Jacobson MD (1993) Programmed cell death and the control of cell survival: lessons from the nervous system. *Science* 262: 695-700.

Ramachandran B, Houben K, Rozenberg YY, Haigh JR, Varpetian A, Howard BD (1993) Differential expression of transporters for norepinephrine and glutamate in wild type, variant, and WNT1-expressing PC12 cells. *J Biol Chem* 268: 23891-7.

Raman IM, Zhang S, Trussell LO (1994) Pathway-specific variants of AMPA receptors and their contribution to neuronal signaling. *J Neurosci* 14: 4998-5010.

Ransom BR, Fern R (1996) Anoxic-ischemic glial cell injury: mechanisms and consequences. In: *Tissue Oxygen Deprivation* (G. G. Haddad and G. Lister ed), pp 617-652. New York: Marcel Dekker.

Ransom BR, Walz W, Davis PK, Carlini WG (1992) Anoxia-induced changes in extracellular K^+ and pH in mammalian central white matter. *J. Cereb. Blood Flow Metab.* 12: 593-602.

Ransom BR, Waxman SG, Davis PK (1990) Anoxic injury of CNS white matter: protective effect of ketamine. *Neurology* 40: 1399-1403.

Ratan RR, Murphy TH, Baraban JM (1994a) Macromolecular synthesis inhibitors prevent oxidative stress-induced apoptosis in embryonic cortical neurons by shunting cysteine from protein synthesis to glutathione. *J Neurosci* 14: 4385-92.

Ratan RR, Murphy TH, Baraban JM (1994b) Oxidative stress induces apoptosis in embryonic cortical neurons. *J Neurochem* 62: 376-9.

Rauen T, Kanner BI (1994) Localization of the glutamate transporter GLT-1 in rat and macaque monkey retinæ. *Neurosci Lett* 169: 137-40.

Reist NE, Smith SJ (1992) Neurally evoked calcium transients in terminal Schwann cells at the neuromuscular junction. *Proc Natl Acad Sci U S A* 89: 7625-9.

Reiter RJ (1998) Oxidative damage in the central nervous system: protection by melatonin. *Prog Neurobiol* 56: 359-84.

Ren Y, Stys PK (1999) Calcium imaging in live rat optic nerve myelinated axons in vitro using confocal laser microscopy. *Soc Neurosci Abstr* 25: 1509.

Rink A, Fung KM, Trojanowski JQ, Lee VM, Neugebauer E, McIntosh TK (1995) Evidence of apoptotic cell death after experimental traumatic brain injury in the rat. *Am J Pathol* 147: 1575-83.

Rivlin AS, Tator CH (1978) Effect of duration of acute spinal cord compression in a new acute cord injury model in the rat. *Surg Neurol* 10: 38-43.

Robinson MB, Dowd LA (1997) Heterogeneity and functional properties of subtypes of sodium-dependent glutamate transporters in the mammalian central nervous system. *Adv Pharmacol* 37: 69-115.

Roche KW, O'Brien RJ, Mammen AL, Bernhardt J, Huganir RL (1996) Characterization of multiple phosphorylation sites on the AMPA receptor GluR1 subunit. *Neuron* 16: 1179-88.

Rosenberg LJ, Teng YD, Wrathall JR (1999a) 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f)quinoxaline reduces glial loss and acute white matter pathology after experimental spinal cord contusion. *J Neurosci* 19: 464-475.

Rosenberg LJ, Teng YD, Wrathall JR (1999b) Effects of the sodium channel blocker tetrodotoxin on acute white matter pathology after experimental contusive spinal cord injury. *J Neurosci* 19: 6122-33.

Rosenmund C, Stern-Bach Y, Stevens CF (1998) The tetrameric structure of a glutamate receptor channel. *Science* 280: 1596-9.

Rossi DJ, Oshima T, Attwell D (2000) Glutamate release in severe brain ischaemia is mainly by reversed uptake. *Nature* 403: 316-321.

Rothman SM, Olney JW (1995) Excitotoxicity and the NMDA receptor--still lethal after eight years. *Trends Neurosci* 18: 57-8.

Rothstein JD, Dykes-Hoberg M, Pardo CA, Bristol LA, Jin L, Kuncl RW, Kanai Y, Hediger MA, Wang Y, Schielke JP, Welty DF (1996) Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate. *Neuron* 16: 675-86.

Rothstein JD, Martin L, Levey AI, Dykes-Hoberg M, Jin L, Wu D, Nash N, Kuncl RW (1994) Localization of neuronal and glial glutamate transporters. *Neuron* 13: 713-725.

Rutledge EM, Kimelberg HK (1996) Release of [3H]-D-aspartate from primary astrocyte cultures in response to raised external potassium. *J Neurosci* 16: 7803-11.

Sakatani K, Hassan AZ, Ching W (1991) Age-dependent extrasynaptic modulation of axonal conduction by exogenous and endogenous GABA in the rat optic nerve. *Exp Neurol* 114: 307-14.

Salzer JL (1997) Clustering of sodium channels at the node of Ranvier: close encounters of the axon-glia kind. *Neuron* 18: 843-846.

Sandler AN, Tator CH (1976) Review of the effect of spinal cord trauma on the vessels and blood flow in the spinal cord. *J Neurosurg* 45: 638-46.

Saruhashi Y, Young W, Sugimori M, Abrahams J, Sakuma J (1999) GABA increases refractoriness of adult rat dorsal column axons. *Neuroscience* 94: 1207-12.

Savidge JR, Bristow DR (1998) Ca^{2+} permeability and joro spider toxin sensitivity of AMPA and kainate receptors on cerebellar granule cells. *Eur J Pharmacol* 351: 131-8.

Schinder AF, Olson EC, Spitzer NC, Montal M (1996) Mitochondrial dysfunction is a primary event in glutamate neurotoxicity. *J Neurosci* 16: 6125-33.

Schousboe A, Svenneby G, Hertz L (1977) Uptake and metabolism of glutamate in astrocytes cultured from dissociated mouse brain hemispheres. *J Neurochem* 29: 999-1005.

Schroder UH, Opitz T, Jager T, Sabelhaus CF, Breder J, Reymann KG (1999) Protective effect of group I metabotropic glutamate receptor activation against hypoxic/hypoglycemic injury in rat hippocampal slices: timing and involvement of protein kinase C. *Neuropharmacology* 38: 209-16.

Schumacher PA, Eubanks JH, Fehlings MG (1999) Increased calpain I-mediated proteolysis, and preferential loss of dephosphorylated NF200, following traumatic spinal cord injury. *Neuroscience* 91: 733-44.

Schumacher PA, Siman RG, Fehlings MG (2000) Pretreatment with calpain inhibitor CEP-4143 inhibits calpain I activation and cytoskeletal degradation, improves neurological function, and enhances axonal survival after traumatic spinal cord injury. *J Neurochem* 74: 1646-55.

Seeburg PH (1993) The TiPS/TINS lecture: the molecular biology of mammalian glutamate receptor channels. *Trends Pharmacol Sci* 14: 297-303.

Seeburg PH, Higuchi M, Sprengel R (1998) RNA editing of brain glutamate receptor channels: mechanism and physiology. *Brain Res Brain Res Rev* 26: 217-29.

Seifert G, Steinhauser C (1995) Glial cells in the mouse hippocampus express AMPA receptors with an intermediate Ca^{2+} permeability. *Eur J Neurosci* 7: 1872-81.

Sekiguchi M, Fleck MW, Mayer ML, Takeo J, Chiba Y, Yamashita S, Wada K (1997) A novel allosteric potentiator of AMPA receptors: 4--2- (phenylsulfonylamino)ethylthio--2,6-difluoro-phenoxyaceta mide. *J Neurosci* 17: 5760-71.

Shashidharan P, Plaitakis A (1993) Cloning and characterization of a glutamate transporter cDNA from human cerebellum. *Biochim Biophys Acta* 1216: 161-4.

Shashidharan P, Wittenberg I, Plaitakis A (1994) Molecular cloning of human brain glutamate/aspartate transporter II. *Biochim Biophys Acta* 1191: 393-6.

Sheardown MJ, Neilsen EO, Hansen AJ, Jacobsen P, Honoré T (1990) 2,3-Dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline: a neuroprotectant for cerebral ischemia. *Science* 247: 571-574.

Sheardown MJ, Suzdak PD, Nordholm L (1993) AMPA, but not NMDA, receptor antagonism is neuroprotective in gerbil global ischaemia, even when delayed 24 h. *Eur J Pharmacol* 236: 347-53.

Shuman SL, Bresnahan JC, Beattie MS (1997) Apoptosis of microglia and oligodendrocytes after spinal cord contusion in rats. *J Neurosci Res* 50: 798-808.

Siesjö BK (1986) Calcium and ischemic brain damage. *Eur Neurol* 25: 45-56.

Siesjö BK, Elmer E, Janelidze S, Keep M, Kristian T, Ouyang YB, Uchino H (1999) Role and mechanisms of secondary mitochondrial failure. *Acta Neurochir Suppl* 73: 7-13.

Siesjo BK, Siesjo P (1996) Mechanisms of secondary brain injury. *Eur J Anaesthesiol* 13: 247-68.

Simpson PB, Russell JT (1998) Role of mitochondrial Ca^{2+} regulation in neuronal and glial cell signalling. *Brain Res Brain Res Rev* 26: 72-81.

Slotboom DJ, Konings WN, Lolkema JS (1999) Structural features of the glutamate transporter family. *Microbiol Mol Biol Rev* 63: 293-307.

Smith DH, Okiyama K, Thomas MJ, McIntosh TK (1993) Effects of the excitatory amino acid receptor antagonists kynurenate and indole-2-carboxylic acid on behavioral and neurochemical outcome following experimental brain injury. *J Neurosci* 13: 5383-92.

Sommer B, Keinänen K, Verdoorn TA, Wisden W, Burnashev N, Herb A, Kohler M, Takagi T, Sakmann B, Seeburg PH (1990) Flip and flop: a cell-specific functional switch in glutamate-operated channels of the CNS. *Science* 249: 1580-5.

Sommer B, Kohler M, Sprengel R, Seeburg PH (1991) RNA editing in brain controls a determinant of ion flow in glutamate-gated channels. *Cell* 67: 11-9.

Sontheimer H, Black JA, Waxman SG (1996) Voltage-gated Na^{+} channels in glia: properties and possible functions. *Trends Neurosci* 19: 325-31.

Staubli U, Chun D (1996) Factors regulating the reversibility of long-term potentiation. *J Neurosci* 16: 853-60.

Staubli U, Rogers G, Lynch G (1994) Facilitation of glutamate receptors enhances memory. *Proc Natl Acad Sci U S A* 91: 777-81.

Steffensen I, Waxman SG, Mills L, Stys PK (1997) Immunohistochemical localization of the Na^+ - Ca^{2+} exchanger in rat central and peripheral myelinated axons. *Brain Res* 776: 1-9.

Steinhauser C, Gallo V (1996) News on glutamate receptors in glial cells. *Trends Neurosci* 19: 339-45.

Stern-Bach Y, Bettler B, Hartley M, Sheppard PO, O'Hara PJ, Heinemann SF (1994) Agonist selectivity of glutamate receptors is specified by two domains structurally related to bacterial amino acid-binding proteins. *Neuron* 13: 1345-57.

Stern-Bach Y, Russo S, Neuman M, Rosenmund C (1998) A point mutation in the glutamate binding site blocks desensitization of AMPA receptors. *Neuron* 21: 907-18.

Storck T, Schulte S, Hofmann K, Stoffel W (1992) Structure, expression, and functional analysis of a Na^{+} -dependent glutamate/aspartate transporter from rat brain. *Proc Natl Acad Sci U S A* 89: 10955-9.

Stout AK, Raphael HM, Kanterewicz BI, Klann E, Reynolds IJ (1998) Glutamate-Induced Neuron Death Requires Mitochondrial Calcium Uptake. *Nature Neurosci* 1: 366-373.

Stys PK (1995) Protective effects of antiarrhythmic agents against anoxic injury in CNS white matter. *J Cereb Blood Flow Metab* 15: 425-432.

Stys PK (1998) Anoxic and ischemic injury of myelinated axons in CNS white matter: from mechanistic concepts to therapeutics. *J Cereb Blood Flow Metab* 18: 2-25.

Stys PK, Lesiuk H (1996) Correlation between electrophysiological effects of mexiletine and ischemic protection of CNS white matter. *Neuroscience* 71: 27-36.

Stys PK, Li S (2000) Glutamate-induced white matter injury: excitotoxicity without synapses. *The Neuroscientist* 6: 235-8.

Stys PK, LoPachin RM (1996) Elemental composition and water content of rat optic nerve myelinated axons during in vitro post-anoxia reoxygenation. *Neuroscience* 73: 1081-1090.

Stys PK, LoPachin RM (1998) Mechanisms of ion flux in anoxic myelinated CNS axons. *Neuroscience* 82: 21-32.

Stys PK, Ransom BR, Black JA, Waxman SG (1995) Anoxic-ischemic injury in axons. In: The Axon: Structure, Function and Pathophysiology (S. G. Waxman, J. D. Kocsis and P. K. Stys ed), pp 462-479. New York: Oxford University Press.

Stys PK, Ransom BR, Waxman SG (1992a) Tertiary and quaternary local anesthetics protect CNS white matter from anoxic injury at concentrations that do not block excitability. J Neurophysiol 67: 236-240.

Stys PK, Ransom BR, Waxman SG, Davis PK (1990) Role of extracellular calcium in anoxic injury of mammalian central white matter. Proc Natl Acad Sci USA 87: 4212-4216.

Stys PK, Sontheimer H, Ransom BR, Waxman SG (1993) Non-inactivating, TTX-sensitive Na^+ conductance in rat optic nerve axons. Proc Natl Acad Sci USA 90: 6976-6980.

Stys PK, Waxman SG, Ransom BR (1992b) Ionic mechanisms of anoxic injury in mammalian CNS white matter: role of Na^+ channels and $\text{Na}^+ - \text{Ca}^{2+}$ exchanger. J Neurosci 12: 430-439.

Swanson GT, Kamboj SK, Cull-Candy SG (1997) Single-channel properties of recombinant AMPA receptors depend on RNA editing, splice variation, and subunit composition. *J Neurosci* 17: 58-69.

Sykova E, Svoboda J, Simonova Z, Jendelova P (1992) Role of astrocytes in ionic and volume homeostasis in spinal cord during development and injury. *Prog Brain Res* 94: 47-56.

Szabo C, Zingarelli B, O'Connor M, Salzman AL (1996) DNA strand breakage, activation of poly (ADP-ribose) synthetase, and cellular energy depletion are involved in the cytotoxicity of macrophages and smooth muscle cells exposed to peroxynitrite. *Proc Natl Acad Sci U S A* 93: 1753-8.

Szatkowski M, Attwell D (1994) Triggering and execution of neuronal death in brain ischaemia: two phases of glutamate release by different mechanisms. *Trends Neurosci* 17: 359-65.

Szatkowski M, Barbour B, Attwell D (1990) Non-vesicular release of glutamate from glial cells by reversed electrogenic glutamate uptake. *Nature* 348: 443-6.

Tachibana M, Okada T (1991) Release of endogenous excitatory amino acids from ON-type bipolar cells isolated from the goldfish retina. *J Neurosci* 11: 2199-208.

Takahashi M, Billups B, Rossi D, Sarantis M, Hamann M, Attwell D (1997) The role of glutamate transporters in glutamate homeostasis in the brain. *J Exp Biol* 200: 401-9.

Takuma K, Matsuda T, Hashimoto H, Asano S, Baba A (1994) Cultured rat astrocytes possess Na⁺-Ca²⁺ exchanger. *Glia* 12: 336-342.

Taoka Y, Okajima K (1998) Spinal cord injury in the rat. *Prog Neurobiol* 56: 341-58.

Tator CH (1991) Review of experimental spinal cord injury with emphasis on the local and systemic circulatory effects. *Neurochirurgie* 37: 291-302.

Tator CH, Koyanagi I (1997) Vascular mechanisms in the pathophysiology of human spinal cord injury. *J Neurosurg* 86: 483-492.

Teichberg VI (1991) Glial glutamate receptors: likely actors in brain signaling. *FASEB J* 5: 3086-91.

Teng YD, Wrathall JR (1997) Local blockade of sodium channels by tetrodotoxin ameliorates tissue loss and long-term functional deficits resulting from experimental spinal cord injury. *J Neurosci* 17: 4359-4366.

Thompson CB (1995) Apoptosis in the pathogenesis and treatment of disease. *Science* 267: 1456-62.

Thornberry NA, Lazebnik Y (1998) Caspases: enemies within. *Science* 281: 1312-6.

Tingley WG, Roche KW, Thompson AK, Huganir RL (1993) Regulation of NMDA receptor phosphorylation by alternative splicing of the C-terminal domain. *Nature* 364: 70-3.

Torp R, Danbolt NC, Babaie E, Bjoras M, Seeberg E, Storm-Mathisen J, Ottersen OP (1994) Differential expression of two glial glutamate transporters in the rat brain: an in situ hybridization study. *Eur J Neurosci* 6: 936-42.

Trussell LO, Zhang S, Raman IM (1993) Desensitization of AMPA receptors upon multiquantal neurotransmitter release. *Neuron* 10: 1185-96.

Turetsky DM, Canzoniero LM, Sensi SL, Weiss JH, Goldberg MP, Choi DW (1994) Cortical neurones exhibiting kainate-activated Ca^{2+} uptake are selectively vulnerable to AMPA/kainate receptor-mediated toxicity. *Neurobiol Dis* 1: 101-10.

Turrens JF (1997) Superoxide production by the mitochondrial respiratory chain. *Biosci Rep* 17: 3-8.

Turrens JF, Alexandre A, Lehninger AL (1985) Ubisemiquinone is the electron donor for superoxide formation by complex III of heart mitochondria. *Arch Biochem Biophys* 237: 408-14.

Turski L, Huth A, Sheardown M, McDonald F, Neuhaus R, Schneider HH, Dirnagl U, Wiegand F, Jacobsen P, Ottow E (1998) ZK200775: a phosphonate quinoxalinedione AMPA antagonist for neuroprotection in stroke and trauma. *Proc Natl Acad Sci U S A* 95: 10960-5.

Tymianski M, Tator CH (1996) Normal and abnormal calcium homeostasis in neurons: a basis for the pathophysiology of traumatic and ischemic central nervous system injury. *Neurosurgery* 38: 1176-95.

Uchino S, Sakimura K, Nagahari K, Mishina M (1992) Mutations in a putative agonist binding region of the AMPA-selective glutamate receptor channel. *FEBS Lett* 308: 253-7.

Vandenberg RJ (1998) Molecular pharmacology and physiology of glutamate transporters in the central nervous system. *Clin Exp Pharmacol Physiol* 25: 393-400.

Vergun O, Keelan J, Khodorov BI, Duchen MR (1999) Glutamate-induced mitochondrial depolarisation and perturbation of calcium homeostasis in cultured rat hippocampal neurones. *J Physiol (Lond)* 519 Pt 2: 451-66.

Verkhratsky A, Kettenmann H (1996) Calcium signalling in glial cells. Trends Neurosci 19: 346-52.

Verkhratsky A, Orkand RK, Kettenmann H (1998) Glial calcium: homeostasis and signaling function. Physiol Rev 78: 99-141.

Vink R, Faden AI, McIntosh TK (1988) Changes in cellular bioenergetic state following graded traumatic brain injury in rats: determination by phosphorus 31 magnetic resonance spectroscopy. J Neurotrauma 5: 315-30.

Wachtler J, Mayer C, Grafe P (1998) Activity-dependent intracellular Ca²⁺ transients in unmyelinated nerve fibers of the isolate adult rat vagus nerve. Pflugers Arch. 435: 678-686.

Wadiche JI, Amara SG, Kavanaugh MP (1995a) Ion fluxes associated with excitatory amino acid transport. Neuron 15: 721-8.

Wadiche JI, Arriza JL, Amara SG, Kavanaugh MP (1995b) Kinetics of a human glutamate transporter. Neuron 14: 1019-27.

Wang GJ, Randall RD, Thayer SA (1994) Glutamate-induced intracellular acidification of cultured hippocampal neurons demonstrates altered energy metabolism resulting from Ca^{2+} loads. *J Neurophysiol* 72: 2563-9.

Wang KK (2000) Calpain and caspase: can you tell the difference? *Trends Neurosci* 23: 20-6.

Washburn MS, Numberger M, Zhang S, Dingledine R (1997) Differential dependence on GluR2 expression of three characteristic features of AMPA receptors. *J Neurosci* 17: 9393-406.

Waters DJ, Allen TG (1998) Ca^{2+} -permeable non-NMDA glutamate receptors in rat magnocellular basal forebrain neurones. *J Physiol (Lond)* 508: 453-69.

Waxman SG (1995) Voltage-gated ion channels in axons: localization, function, and development. In: *The Axon: Structure, Function and Pathophysiology* (S. G. Waxman, J. D. Kocsis and P. K. Stys ed), pp 218-243. New York: Oxford University Press.

Waxman SG, Black JA, Ransom BR, Stys PK (1993) Protection of the axonal cytoskeleton in anoxic optic nerve by decreased extracellular calcium. *Brain Res.* 614: 137-145.

Waxman SG, Black JA, Stys PK, Ransom BR (1992) Ultrastructural concomitants of anoxic injury and early post-anoxic recovery in rat optic nerve. *Brain Res.* 574: 105-119.

Waxman SG, Ritchie JM (1993) Molecular dissection of the myelinated axon. *Ann. Neurol.* 33: 121-136.

White RJ, Reynolds IJ (1996) Mitochondrial depolarization in glutamate-stimulated neurons: an early signal specific to excitotoxin exposure. *J Neurosci* 16: 5688-97.

Wisden W, Seeburg PH (1993) Mammalian ionotropic glutamate receptors. *Curr Opin Neurobiol* 3: 291-8.

Wrathall JR, Bouzoukis J, Choiniere D (1992) Effect of kynurenate on functional deficits resulting from traumatic spinal cord injury. *Eur J Pharmacol* 218: 273-81.

Wrathall JR, Choiniere D, Teng YD (1994) Dose-dependent reduction of tissue loss and functional impairment after spinal cord trauma with the AMPA/kainate antagonist NBQX. *J Neurosci* 14: 6598-6607.

Wrathall JR, Teng YD, Marriott R (1997) Delayed antagonism of AMPA/kainate receptors reduces long-term functional deficits resulting from spinal cord trauma. *Exp Neurol* 145: 565-73.

Wright M, Rivara FP, Ferse D (1995) Evaluation of the Think First head and spinal cord injury prevention program. *Inj Prev* 1: 81-5.

Yamada K, Watanabe M, Shibata T, Tanaka K, Wada K, Inoue Y (1996) EAAT4 is a post-synaptic glutamate transporter at Purkinje cell synapses. *Neuroreport* 7: 2013-7.

Yamada KA, Rothman SM (1992) Diazoxide blocks glutamate desensitization and prolongs excitatory postsynaptic currents in rat hippocampal neurons. *J Physiol (Lond)* 458: 409-23.

Yamamoto-Hino M, Miyawaki A, Kawano H, Sugiyama T, Furuichi T, Hasegawa M, Mikoshiba K (1995) Immunohistochemical study of inositol 1,4,5-trisphosphate receptor type 3 in rat central nervous system. *Neuroreport* 6: 273-6.

Yatsugi S, Takahashi M, Kawasaki-Yatsugi S, Koshiya K, Sakamoto S, Uematsu D, Shimizu-Sasamata M (1996) Neuroprotective effect of YM90K, a novel AMPA/kainate receptor antagonist, in focal cerebral ischemia in cats. *J Cereb Blood Flow Metab* 16: 959-66.

Yong C, Arnold PM, Zoubine MN, Citron BA, Watanabe I, Berman NE, Festoff BW (1998) Apoptosis in cellular compartments of rat spinal cord after severe contusion injury. *J Neurotrauma* 15: 459-72.

Yoshioka A, Hardy M, Younkin DP, Grinspan JB, Stern JL, Pleasure D (1995) Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors mediate excitotoxicity in the oligodendroglial lineage. *J Neurochem* 64: 2442-8.

Young W (1996) Spinal cord injury pathophysiology and therapy. In: *Neurotrauma* (R. K. Narayan, J. E. Wilberger Jr. and J. T. Povlishock ed), pp 1075-1093. New York: McGraw-Hill.

Young W, Kume-Kick J, Constantini S (1994) Glucocorticoid therapy of spinal cord injury. *Ann N Y Acad Sci* 743: 241-63.

Zerangue N, Kavanaugh MP (1996) Flux coupling in a neuronal glutamate transporter. *Nature* 383: 634-637.

Zhang D, Sucher NJ, Lipton SA (1995) Co-expression of AMPA/kainate receptor-operated channels with high and low Ca^{2+} permeability in single rat retinal ganglion cells. *Neuroscience* 67: 177-88.

Zimmerman UP, Schlaepfer WW (1984) Calcium-activated neutral protease (CANP) in brain and other tissues. *Prog Neurobiol* 23: 63-78.