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Novel Injury Mechanism in Anoxia and Trauma of Spinal Cord White Matter: Glutamate Release via Reverse Na[1]-dependent Glutamate Transport

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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 sec of mechanical compression at a force of 2 gm *in vitro*. The broad-spectrum glutamate antagonist kynurenic acid (1 mM) and the selective AMPA antagonist GYKI52466 (30 mM) 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[1]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 axon cylinders and oligodendrocytes 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[1]dependent glutamate transport with subsequent activation of AMPA receptors is an important mechanism in spinal cord white matter anoxic and traumatic injury.

*Key words: spinal cord injury; axon; anoxia; trauma; AMPA; Na[1]-glutamate transport; myelin; dihydrokainate; L-trans*pyrrolidine-2,4-dicarboxylic acid; GYKI52466; kynurenic acid

White matter tracts within the mammalian 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 .10,000 new cases per year occurring in the United States alone (Gibson, 1992). Although 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 to devise more effective treatments for the acute phase.

Recent reports using *in vitro* and *in vivo* SCI models indicate that voltage-gated Na[1] 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 (Stys et al., 1992; Imaizumi et al., 1997). In addition, injury is also dependent on excitotoxic mechanisms involving AMPA and 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[1]-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 either Na[1] channel blockade or AMPA antagonists are neuroprotective in SCI, because Na[1] 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[1], zero-Ca[21] solution. A 30 mm section of spinal cord was rapidly removed and placed in cold (4–6 °C)

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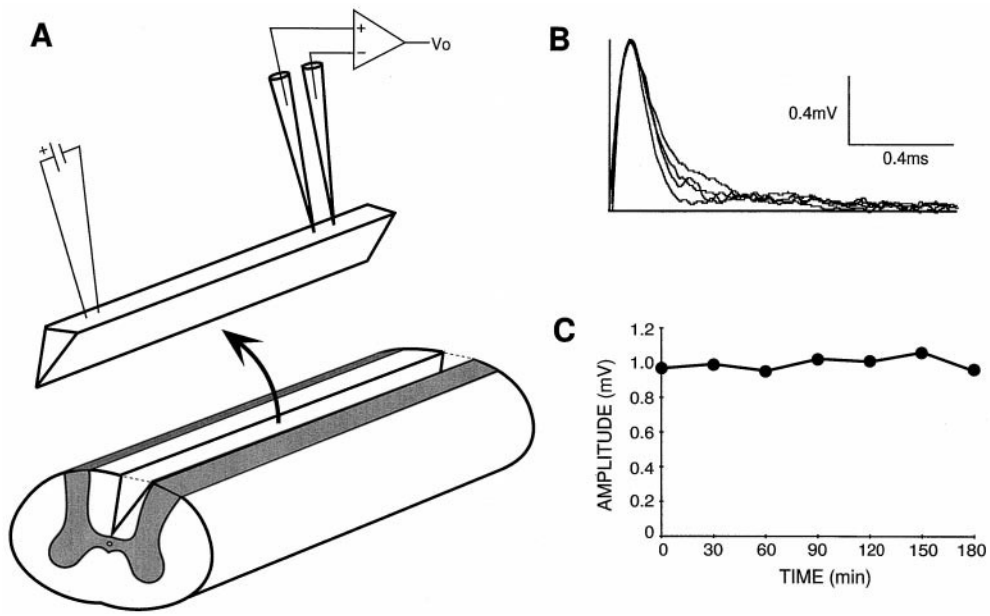


Figure 1. A, Schematic of recording arrangement. Dorsal column slices were incubated in an *in vitro* recording chamber. Stimulating and recording surface electrodes were used to evoke compound propagated action potentials. *B,* Representative tracings of compound action potentials shown at intervals of 1 hr recorded over 3 hr 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 Materials and Methods). *C,* Graph quantitatively showing stable peak amplitudes over 3 hr at 37°C.

zero-Na^1, zero-Ca^{21} solution bubbled with 95% O_2 and 5% CO_2. Dorsal column sections were excised and placed in an interface recording chamber bathed in Ca^{21}-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 $MgSO_4$, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 2.0 $CaCl_2$, 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 msec 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 (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 $CaCl_2$, 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. Wholecell currents were measured at a holding potential of 260 mV after a 0.5 sec application of cyclothiazide (100 mM), then 1 sec AMPA (100 mM) 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 dorsal column injury and continued until 15 min after injury, after which tissue was washed with aCSF. Injury was induced by anoxia or trauma. Anoxia was achieved by switching to a 95% N_2 and 5% CO_2 atmosphere for 60 min and then reoxygenating for 2 hr. Trauma was induced by compression with a custom-made aneurysm clip calibrated to a closing force of 2 gm (David Walsh, Oakville, Ontario, Canada), applied for 15 sec between the stimulation and recording sites (Agrawal and Fehlings, 1997). GYKI52466 (Research Biochemicals, Natick, MA), dihydrokainic acid, and L-*trans*-pyrrolidine-2,4-dicarboxylic were dissolved in 0.1N HCI (GYKI52466) or 0.1N NaOH and then added to aCSF to the desired final concentration. Kynurenic acid (Research Biochemicals) 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), 2939cyclic nucleotide 39-phosphohydrolase (CNPase, Chemicon), and antiglial 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 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% paraformaldedyde 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 mg/ml for anti-GLT1 (N terminus; Alpha Diagnostic International, San Antonio, TX), 0.98 mg/ml for anti-GLAST (N terminus), 0.49 mg/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 603 oil-immersion lens (Olympus Optical, Tokyo, Japan).

Statistics. All data are expressed as means 6 SD. 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.

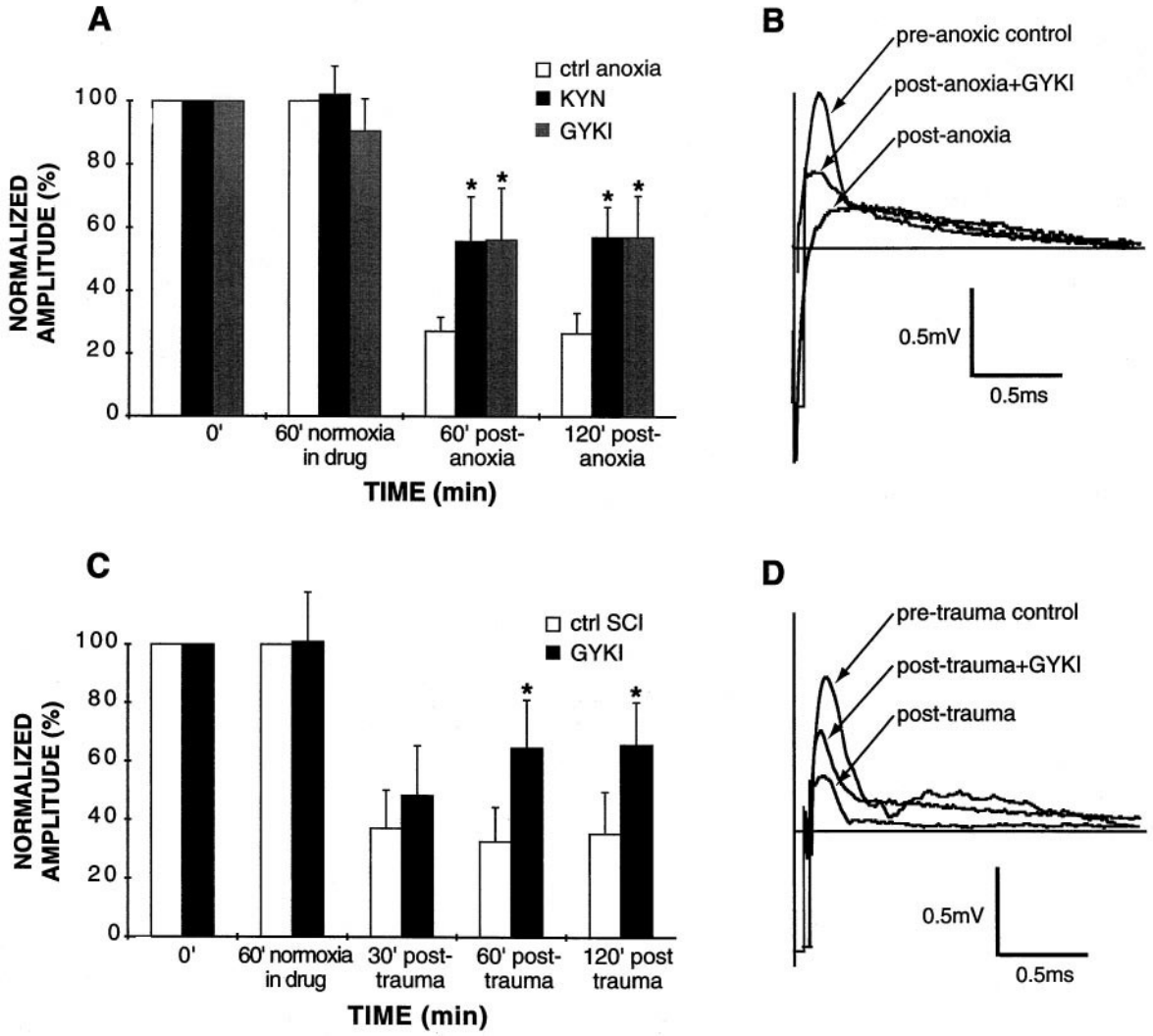


Figure 2. Bar graphs illustrating recovery of compound action potential amplitudes recorded from dorsal column slices *in vitro* after 60 min of anoxia (*A*) or 15 sec of traumatic compression at 2 gm (*C*). Peak amplitudes were normalized to baseline responses recorded at time 0 (see Materials and Methods). 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 were measured at 60 and 120 min of reoxygenation (*60*9 and *120*9 *post-anoxia bars*). In aCSF alone, amplitudes recovered to ;27% of preanoxic control (*ctrl anoxia*; *n* 5 12), whereas recovery was significantly enhanced to ;55% of control by kynurenic acid (*KYN*, 1 mM; *n* 5 6) or GYKI52466 (*GYKI*, 30 mM; *n* 5 7) (*A*). Similarly, GYKI52466 (*n* 5 14) improved recovery after trauma from 35% (*ctrl SCI*; *n* 5 13) to 65% of preinjury control amplitude. *B*, *D*, Representative compound action potential tracings obtained after 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 with time-matched readings of slices injured in the absence of drug.

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 1 mM 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 mM), 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. 3*A*) and tissue injured by anoxia (Fig. 3*B–E*) or SCI (Fig. 3*F*,*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[1]-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

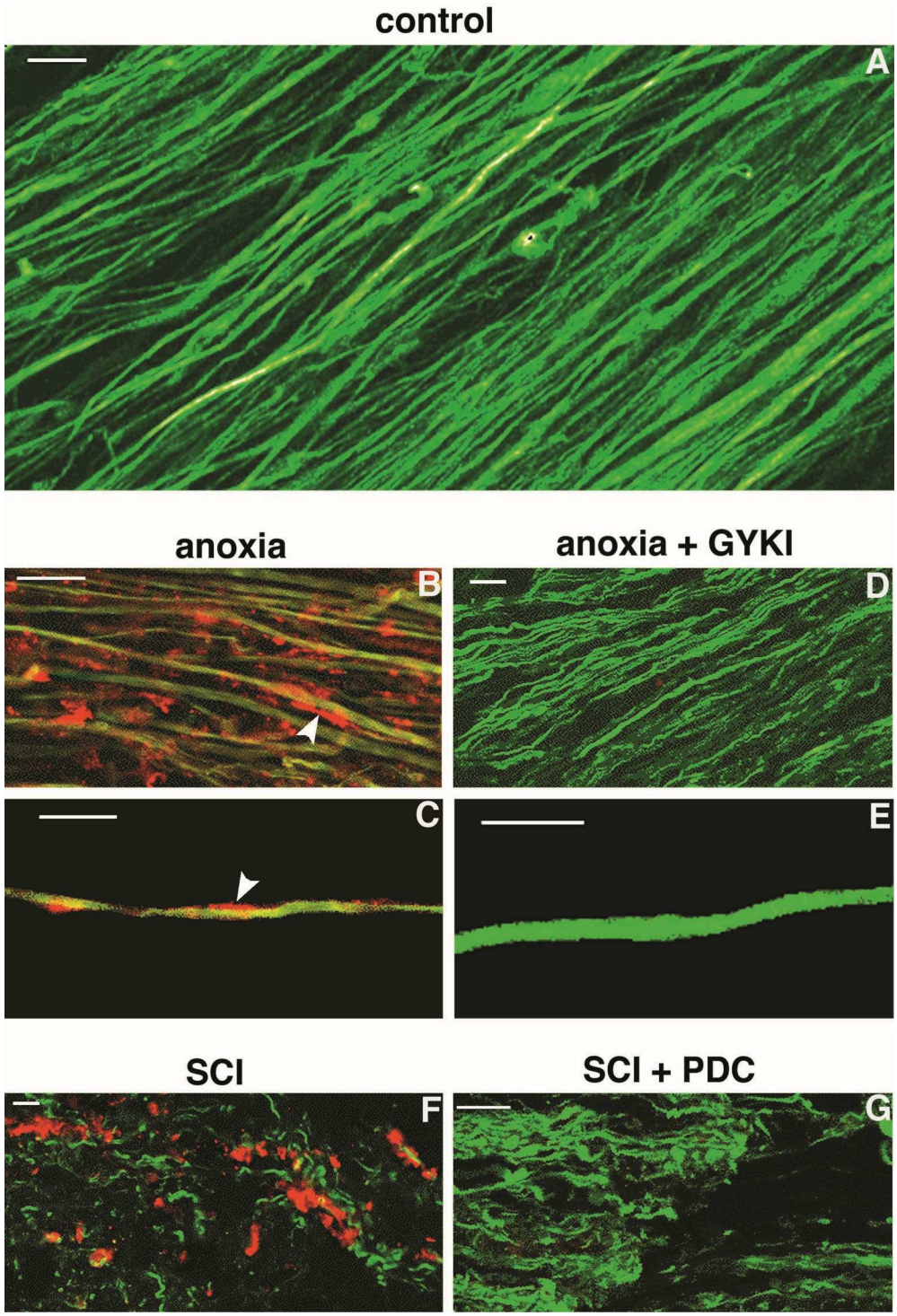


Figure 3. Immunohistochemistry of dorsal column axons stained for neurofilament (*green*) outlining axon cylinders and damaged myelin detected by antiserum raised against degenerated myelin basic protein (*red*). *A*, Control sections show virtually no myelin damage. A 60 min anoxic exposure caused significant myelin damage as shown by strong immunoreactivity surrounding many axon cylinders (*B*, *C*, *arrowheads*). *C*, Higher-power view of a single damaged axon. The AMPA receptor blocker GYKI52466 greatly reduced the degree of anoxic myelin damage (*D*, *E*). Sections from the injury focus showed that traumatic compression (*SCI*) also resulted in damage to myelin (*F*, *red* signal) as well as disruption of axon cylinders as evidenced by distorted neurofilament profiles. Trauma in the presence of the Na^1-dependent glutamate transport inhibitor L-*trans*-pyrrolidine2,4-dicarboxylic acid (*SCI* 1 *PDC*) significantly reduced myelin injury (*G*). Bars, 10 mm.

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 Ca^{21}independent manner through reversal of Na^1-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-2,4dicarboxylic 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. 4*A*,*B*). The degree of myelin damage after *in vitro* SCI was markedly reduced by gluta-

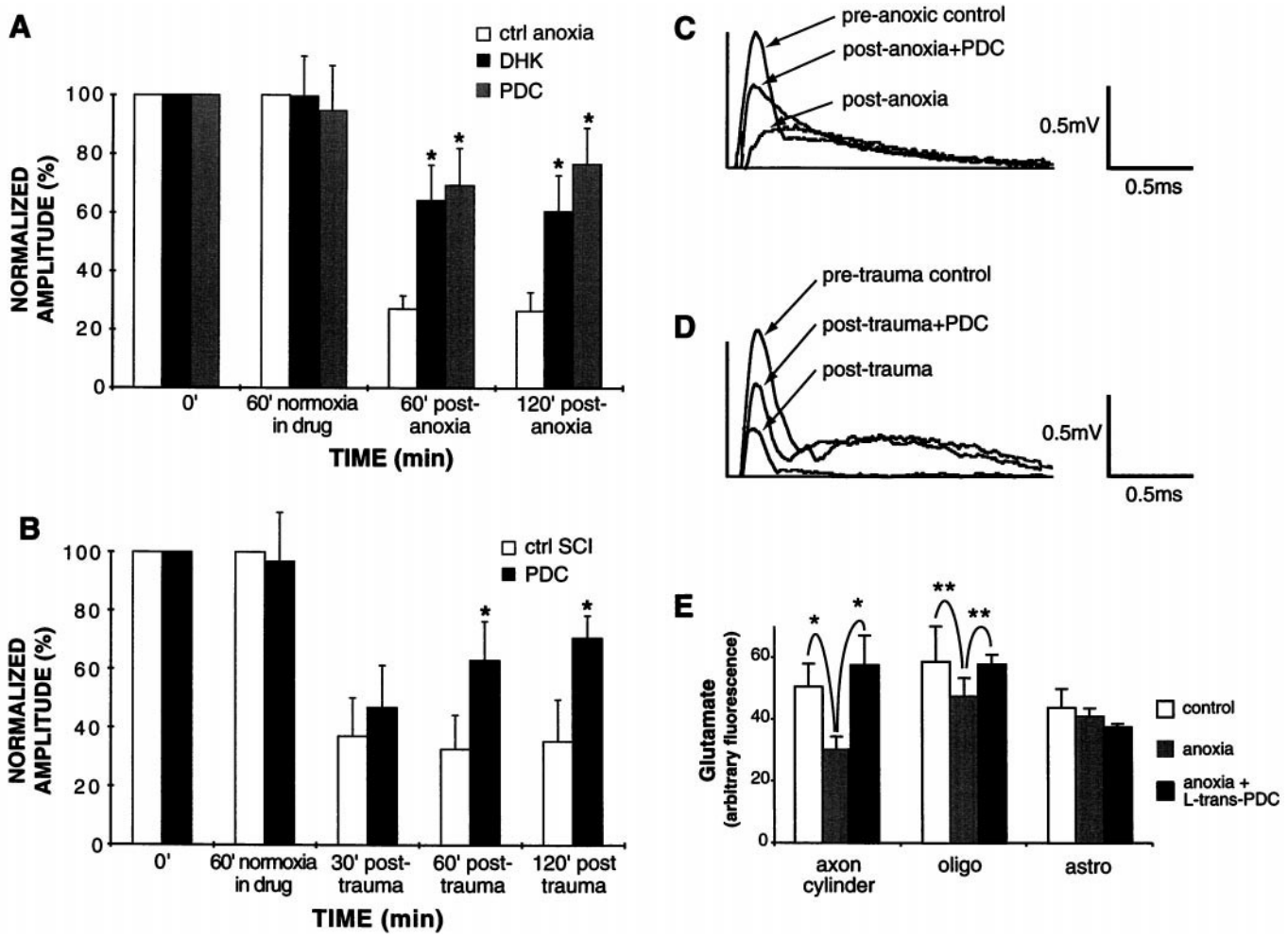


Figure 4. Effect of Na[1]-dependent glutamate transport inhibitors on the recovery of compound action potentials after anoxia or trauma. Drugs were applied beginning 60 min before and continued until 15 min after injury. Neither inhibitor [dihydrokainate (*DHK*) or L-*trans*-pyrrolidine-2,4-dicarboxylic acid (*PDC*), both 1 mM] had any significant effect on preinjury responses (*609 normoxia in drug bars*). Both agents improved compound action potential amplitudes significantly after 60 min of anoxia (*A*) or a 15 sec traumatic compression (*B*). *p , 0.01 compared with time-matched readings of slices injured in the absence of drug. *C*, *D*, Representative compound action potential tracings. *Bar graph* in *E*, Summary of 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[1]-dependent glutamate transporters during anoxic or traumatic injury. (*n* values: *A, ctrl anoxia*, 12; *DHK*, 7; *PDC*, 7; *B, ctrl SCI*, 13, *PDC*, 7; *E*, minimum of 10 images, each containing multiple regions of interest per group).

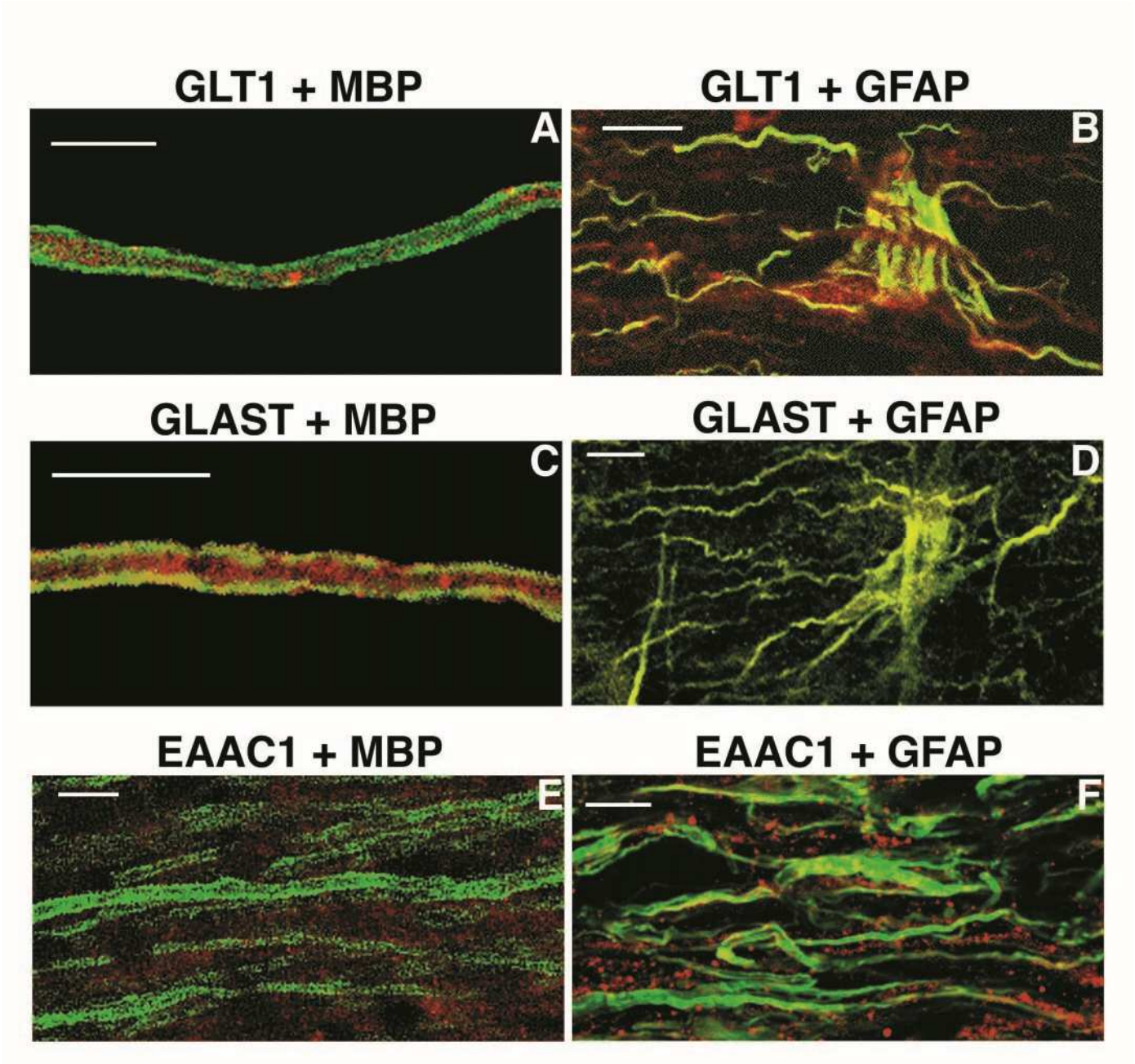
mate transport inhibition (Fig. 3*F*,*G*), although the disruption of axon cylinders shown by neurofilament staining appeared unchanged. Confocal fluorescence was used to estimate semiquantitatively the cytosolic glutamate concentrations in axon cylinders and glial cell bodies and processes after 1 hr of anoxia. Figure 4*E* 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 AMPAinduced currents were measured by patch clamp in cultured neurons. Steady-state currents were increased slightly to 111 6 5 and 120 6 11% of control by 1 mM L-*trans*-pyrrolidine-2,4dicarboxylic acid and dihydrokainate, respectively (data not shown). This was not statistically significant.

Dorsal white matter possesses three subtypes of Na[1] dependent glutamate transporters: GLT1, GLAST, and EAAC1

The previous results provide pharmacological evidence for the presence of Na[1]-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. 5*B*), with fainter stain seen within the axoplasm of myelinated axons (Fig. 5*A*). GLT1 was not found in myelin, in contrast to GLAST, which was present throughout the thickness of the sheath (Fig. 5*C*). GLAST was also observed in astrocytes (Fig. 5*D*). EAAC1, a predominantly neuronal isoform, was not present in myelin, and label was only weakly observed in some GFAP-positive astrocytes (Fig. 5*F*). There was

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-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 mm.

considerable intervening stain outside myelin and astrocytic regions (Fig. 5*E*), 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 and ischemia, trauma, and demyelination. Indeed, these seemingly disparate injury modalities may share common mechanisms; for example, traumatic spinal cord injury consists of 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, in which excitotoxicity leading to Ca^{21}-mediated injury, free radical generation, and delayed apoptosis are thought to be the main avenues by which neurons succumb to anoxia and ischemia. In anoxic central myelinated axons, in contrast, excessive Na^1 influx through noninactivating Na^1 channels causes Ca^{21} overload largely through reverse Na^1–Ca^{21} exchange. The excessive Ca^{21} influx in turn triggers a variety of Ca^{21}-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 and glucose deprivation causes damage to cultured oligodendroglia that is dependent on AMPA and kainate receptors (McDonald et al., 1998). By extension, it may be possible that the myelin sheath itself possesses AMPA and kainate receptors and may be directly susceptible to injury from high concentrations of ambient glutamate released from compromised axon cylinders or glia.

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 and kainate antagonist 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo (f)quinoxaline (NBQX). The neuroprotective effect of AMPA and 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 and kainate receptor activation as evidenced by a partial neuroprotective effect of CNQX or NBQX (Agrawal and Fehlings, 1997). Taken together, these findings implicate AMPA and 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 1 hr anoxic exposure followed by 1 or 2 hr of reoxygenation resulted in irreversible depression of CAP amplitude to 27% of preanoxic control values. This is very close to the level reported in a similar *in vitro* model (Imaizumi et al., 1997). The broadspectrum 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 greater than 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 gm 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 after 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. 3*B,C,F*). These changes were prevented by selective AMPA inhibition, not only indicating that this subtype of ionotropic glutamate receptor contributes to myelin injury, but also pointing 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, because these cells are known to be sensitive to excitotoxic insults mediated by AMPA and 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 (Fonnum, 1984; Attwell et al., 1993). 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^1-dependent glutamate transport (Attwell et al., 1993). The latter pathway transports glutamate or aspartate with Na^1 and H^1 in exchange for K^1 in an electrogenic manner (Zerangue and Kavanaugh, 1996; Levy et al., 1998). It follows that a rise in $[K^1]_o$ and depolarization, along with an increase in $[Na^1]_i$, 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^1 and accumulation of Na^1 (LoPachin and Stys, 1995), with an expected rise in $[K^1]_o$ and depolarization (Ransom et al., 1992; Leppanen and Stys, 1997), stimuli that would strongly favor reversal of Na^1-dependent glutamate transport. This hypothesis was supported by the markedly neuroprotective effects of the transport inhibitors dihydrokainate and L-*trans*-pyrrolidine-2,4dicarboxylic acid. Moreover, immunohistochemistry for glutamate revealed that anoxic axon cylinders, and to a lesser extent oligodendrocytes, are the main source of endogenous glutamate (Fig. 4*E*); the efflux of glutamate from these sources, and by inference the rise in $[glutamate]_o$, was completely prevented by pharmacological inhibition of Na^1-dependent glutamate transport. Notably, astrocytic glutamate was unchanged by anoxia, in keeping with the relative resistance of ionic deregulation by anoxia alone in this cell type (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 $[glutamate]_o$ 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 (Povlishock and Christman, 1995; Maxwell et al., 1997). 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 230 mV (Leppanen and Stys, 1997), $[Na^1]_i$, $[Na^1]_o$, $[K^1]_i$, and $[K^1]_o$ of 100, 150, 15, and 15 mM, respectively (Ransom et al., 1992; LoPachin and Stys, 1995), a ratio of $[H^1]_i$ to $[H^1]_o$ of 2.5 (with both moving proportionally in the acid direction during injury), $[glutamate]_i$ 5 3 mM (Attwell et al., 1993), and a transporter stoichiometry of 3 Na^1, 1 H^1, 1 $glutamate^2$:1 K^1 (Levy et al., 1998), Equation 1 predicts that $[glutamate]_o$ will exceed 230 mM at equilibrium; this ignores any reductions in $[Na^1]_o$, which would steeply push $[glutamate]_o$ 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^1–glutamate transport in trauma as well:

$$\left[\mathrm{Glu}_o\right] = \left[\mathrm{Glu}_i\right]\left[\mathrm{H}_o\right]^{n_H}\left[\mathrm{Na}_o\right]^{n_{Na}}\left[\mathrm{K}_i\right]^{n_K} e^{\frac{FV_m - n_{Na}\mathrm{Na} + n_H\mathrm{H} - n_{Glu}\mathrm{Glu} + n_K\mathrm{K}}{RT n_{Glu}}}, \quad (1)$$

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

Pharmacological evidence for the presence of Na¹-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 (Sutherland et al., 1996; Choi and Chiu, 1997). 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 (Schmitt et al., 1996; Mennerick et al., 1998). 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 immunoelectronmicroscopy.

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 through reversal of Na¹-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.

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