Complex interplay between glutamate receptors and intracellular Ca^{2+} stores during ischaemia in rat spinal cord white matter

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Electrophysiological recordings of propagated compound action potentials (CAPs) and axonal Ca^{2+} measurements using confocal microscopy were used to study the interplay between AMPA receptors and intracellullar Ca^{2+} stores in rat spinal dorsal columns subjected to *in vitro* combined oxygen and glucose deprivation (OGD). Removal of Ca^{2+} or Na^+ from the perfusate was protective after 30 but not 60 min of OGD. TTX was ineffective with either exposure, consistent with its modest effect on ischaemic depolarization. In contrast, AMPA antagonists were very protective, even after 60 min of OGD where $0Ca^{2+}$ + EGTA perfusate was ineffective. Similarly, blocking ryanodine receptor-mediated Ca^{2+} mobilization from internal stores ($0Ca^{2+}$ + nimodipine or $0Ca^{2+}$ + ryanodine), or inositol 1,4,5-trisphosphate (IP_3)-dependent Ca^{2+} release (block of group 1 metabotropic glutamate receptors with 1-aminoindan-1,5-dicarboxylic acid, inhibition of phospholipase C with U73122 or IP_3 receptor block with 2APB; each in $0Ca^{2+}$) were each very protective, with the combination resulting in virtually complete functional recovery after 1 h OGD (97 ± 32% CAP recovery *versus* 4 ± 6% in artificial cerebrospinal fluid). AMPA induced a rise in Ca^{2+} concentration in normoxic axons, which was greatly reduced by blocking ryanodine receptors. Our data therefore suggest a novel and surprisingly complex interplay between AMPA receptors and Ca^{2+} mobilization from intracellular Ca^{2+} stores. We propose that AMPA receptors may not only allow Ca^{2+} influx from the extracellular space, but may also significantly influence Ca^{2+} release from intra-axonal Ca^{2+} stores. In dorsal column axons, AMPA receptor-dependent mechanisms appear to exert a greater influence than voltage-gated Na^+ channels on functional outcome following OGD.

(Resubmitted 7 July 2006; accepted after revision 29 August 2006; first published online 31 August 2006)
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Central nervous system axons have the critical role of transmitting information with high fidelity and reliability between neurons by action potential propagation from the soma to the presynaptic terminals. Axonal disruption often results in serious morbidity, and is a hallmark of a variety of disorders including spinal cord injury, traumatic and ischaemic brain injury and multiple sclerosis. The mechanisms of axo-glial injury are surprisingly complex: in anoxia/ischaemia, for example, a rapid loss of ATP production results in accumulation of axonal Na^+ and loss of K^+, leading to membrane depolarization and intra-axonal Ca^{2+} overload (for review see Stys, 2004). Blockade of TTX-sensitive Na^+ channels during injury is protective in models of optic nerve anoxia/ischaemia (Stys *et al.* 1992; Fern *et al.* 1993; Leppanen & Stys, 1997*b*; Jiang & Stys, 2000), and in spinal cord trauma (Agrawal & Fehlings, 1996; Teng & Wrathall, 1997) and anoxia (Imaizumi *et al.* 1997). One of the consequences of intra-axonal Na^+ accumulation and depolarization is reversal of Na^+-dependent transporters, such as the Na^+–Ca^{2+} exchanger and Na^+-dependent glutamate transporters, leading to Ca^{2+} accumulation, glutamate release and excitotoxic injury, the latter thought to be mediated by ionotropic and metabotropic glutamate receptors (mGluRs). Thus, inhibition of Na^+–Ca^{2+} exchange, glutamate receptors or Na^+-dependent glutamate transport is protective against white matter anoxia and trauma (Agrawal & Fehlings, 1997; Wrathall *et al.* 1997; Li *et al.* 1999; Rosenberg *et al.* 1999; Li & Stys, 2000; Tekkok & Goldberg, 2001). Pathological depolarization also activates voltage-sensitive Ca^{2+} channels; blockade of L-type and N-type channels partially protects against anoxic and traumatic axonal injury (Fern *et al.* 1995; Imaizumi *et al.* 1999; Wolf *et al.* 2001; Ouardouz *et al.* 2003, 2005). Voltage-gated Ca^{2+} channels may also

 DOI: 10.1113/jphysiol.2006.116798

promote axonal Ca^{2+} overload not only by influx of Ca^{2+} from the extracellular space, but also by triggering release of Ca^{2+} from internal stores by a mechanism similar to excitation–contraction coupling in muscle (Ouardouz *et al.* 2003).

Whereas it is now clear that AMPA/kainate receptor blockade is protective in a variety of white matter injury protocols (Agrawal & Fehlings, 1997; Wrathall *et al.* 1997; Li *et al.* 1999; Rosenberg *et al.* 1999; Tekkok & Goldberg, 2001), the precise loci of action of these receptors are not known: glia are likely targets but it is unclear whether axons may respond directly to activation of these receptors. In this study we used an *in vitro* ischaemic protocol of spinal cord dorsal column injury to study different pathways responsible for inducing axonal damage using both electrophysiological recordings of compound action potentials (CAPs) and confocal microscopy to measure changes in intra-axonal Ca^{2+} concentration. Our results reveal a surprisingly complex interaction between AMPA receptor-dependent mechanisms and release of Ca^{2+} from intracellular stores in the induction of white matter ischaemic injury.

Methods

Tissue preparation

All experimental protocols were approved by the institutional animal care committee. Spinal cord dorsal columns were excised from adult Long-Evans male rats (250–350 g) after intracardiac perfusion with cold $0Ca^{2+}$ saline solution in animals deeply anaesthetized using intraperitoneal injection of sodium pentobarbital (35 mg kg^{-1}). Depth of anaesthesia was assessed by response to peripheral pain and corneal reflex (for details see Ouardouz *et al.* 2003). Longitudinal dorsal column slices, measuring approximately 8–10 mm in length × 1 mm thick, were gently dissected from the thoracic spinal cord and placed in an interface recording chamber continuously perfused with artificial cerebrospinal fluid (aCSF) containing (mM): NaCl 126, KCl 3, $MgSO_4$ 2, $NaHCO_3$ 26, NaH_2PO_4 1.25, $CaCl_2$ 2 and dextrose 10, oxygenated with 95% O_2–5% CO_2 and gradually warmed to 37°C for 1 h.

Measurements of propagated CAPs

Suction electrodes were used to evoke CAPs by constant-voltage pulses from one end and for recording from the opposite end. Electrodes were only applied for the time required to take readings at the various time points. Electrode resistance was measured and used to correct any instability of the recorded amplitudes generated by the application and removal of the electrodes as previously described (Stys *et al.* 1991). After recording control CAPs, ischaemia was induced by combined oxygen and glucose deprivation (OGD) for 1 h, by changing to 5% CO_2–95% N_2 atmosphere in the interface chamber and perfusing with a solution bubbled with 5% CO_2–95% N_2 containing 10 mM sucrose in place of glucose. CAP recovery after OGD was recorded 1–3 h after reoxygenation and reperfusion with glucose-containing oxygenated aCSF. Drugs were pre-applied 1 h before and continued for 15 min following OGD. Data are presented as means ± S.D.

Resting membrane potential measurements

Compound resting membrane potentials were recorded from dorsal column *in vitro* using a grease gap chamber at 37°C (for details see Leppanen & Stys, 1997*a*). The width of the gap was ∼2 mm, and given that glial processes typically extend no more than several hundred microns longitudinally in white matter tracts, axonal potentials could therefore be recorded in isolation. Raw baseline gap potentials (V_g) varied from slice to slice (typical range −20 to −40 mV) because of differences in the short circuit factor (Stämpfli, 1954). V_g recordings in aCSF typically stabilized 90 min after insertion into the gap, and thereafter varied by less than 5% over the next hour of recording. To compare responses over time and between different treatments, ratios of V_g values were calculated at different time points with respect to potentials at time 0 (defined as a stable potential baseline before any experimental treatment). Ischaemia was induced chemically using the glycolytic blocker iodoacetate (1 mM) (Sabri & Ochs, 1971) and the mitochondrial inhibitor NaN_3 (2 mM) (Kauppinen & Nicholls, 1986). We found that this method of inducing ischaemia gave more reproducible membrane potential changes than glucose removal and gaseous N_2; in these experiments reperfusion was not needed so the rapid but irreversible effects of iodoacetate were acceptable, and even preferred.

Axonal Ca^{2+} imaging

Freshly excised dorsal columns were loaded with the red dextran-conjugated dye Alexa Fluor 594 as a reference and the dextran-conjugated Ca^{2+} indicator Oregon Green-488 BAPTA-1 (both from Molecular Probes, Eugene, OR, USA) to monitor Ca^{2+} fluorescence changes as previously described (Ouardouz *et al.* 2003). Typically about six different dorsal columns were studied per treatment group. Imaging was done ∼1 mm from the cut ends. As previously demonstrated (Ren *et al.* 2000; Verbny *et al.* 2002), loading dyes in this manner results in exclusive staining of axon cylinders allowing identification of axonal regions of interest in isolation. A Nikon C1 Eclipse microscope equipped with a 60 × 1.0 NA water immersion objective was used to monitor fluorescence in confocal mode. Dyes were excited at 488 and 594 nm with

Ar and He–Ne lasers, respectively. A dichroic mirror centred at 580 nm split the emission into two channels, which were filtered by 540 ± 30 nm and 630 ± 15 nm bandpass filters. Images were acquired every 60 s at 35°C. Ischaemia was induced chemically as for grease gap experiments using the glycolytic blocker iodoacetate and the mitochondrial inhibitor NaN_3; the open configuration of the perfusion chamber mounted on the upright microscope to allow access by the imaging objective made it impossible to adequately maintain a very low O_2 tension, hence the choice of chemical inhibitors. Data are presented as a ratio of green Ca^{2+}-dependent signal against the Ca^{2+}-insensitive red channel (see Fig. 6), then percentage change during ischaemia compared to control was calculated individually for each axon segment using ImageTrak software written by PKS (http://www.ohri.ca/stys/imagetrak). In order to control for possible fluorescence changes of intrinsic flavin adenine dinucleotide (FAD) in response to metabolic inhibition, whose excitation/emission overlaps with our Ca^{2+} indicator, axons were loaded with the red dextran but the green Ca^{2+} dye was omitted. When dorsal columns were then exposed to identical conditions of chemical ischaemia, a 26% decrease in green fluorescence was observed. However, because absolute FAD fluorescence was about one-third as strong as baseline Oregon Green-488 BAPTA-1 signal, this ischaemic drop in FAD emission would cause a $< 10\%$ underestimate in the true rise of the Ca^{2+} signal; the latter was therefore not corrected for the much smaller FAD fluorescence changes seen with metabolic inhibition.

Ca^{2+} imaging in myelin

Ca^{2+} changes in the myelin sheath were measured as recently described (Micu *et al.* 2006). Freshly excised dorsal columns were loaded with the Ca^{2+} indicator X-rhod-1 to monitor Ca^{2+} change and with 3,3-dihexyloxacarbocyanine iodide (DiOC6(3)) (500 nM) to outline myelin. Two-photon excited fluorescence images were collected every minute using a custom-modified Nikon D-Eclipse C1 laser-scanning microscope (two-photon excitation was used because confocal imaging results in excessive photobleaching of the X-rhod-1 indicator). Data are presented as a ratio of red Ca^{2+}-dependent signal against the Ca^{2+}-insensitive green channel, then percentage change during AMPA application compared to control was calculated individually for each myelin segment using ImageTrak.

Immunohistochemistry

Deeply anaesthetized adult rats were perfused with saline then 4% paraformaldehyde in 0.1 M phosphate buffer. Dorsal columns were excised, postfixed, treated with methanol for 30 min on ice, and then blocked with 4% normal goat serum in Tris buffer containing 1% Triton X-100 for 1 h. Primary antibodies (GluR1, GluR2/3 and GluR4, Chemicon International, 1 : 100; NF160, Sigma clone NN18, 1 : 1000) were applied followed by secondary antibodies (Alexa 488 or Texas Red labelled) at 1 : 100 dilution. Slides were imaged on a Nikon C1 confocal laser scanning microscope with a 60×1.4 NA oil immersion objective.

Statistics

Data are presented as mean ± S.D. and statistical significance was assessed using ANOVA with Dunnett's *post hoc* test for multiple comparisons with a common control group, or Tukey's one-way ANOVA for multiple comparisons, unless otherwise indicated.

Results

CAP recovery at 30 versus 60 min OGD

OGD for 30 or 60 min caused severe injury to spinal cord dorsal column axons as assessed by the irreversible decrease of CAP area measured 3 h after the insult, to allow maximal recovery. As shown in Fig. 1, CAPs recovered to $29 \pm 19\%$ of control after 30 min OGD ($n = 8$, Fig. 1*A* and *C*) and to $4 \pm 6\%$ of control following 60 min of OGD ($n = 12$, Fig. 1*B* and *C*). It is interesting to note that the ability of external $0Ca^{2+}$ (with 0.5 mM EGTA) solution to protect dorsal columns against OGD was heavily dependent on duration of the insult: after a 30-min OGD exposure, $0Ca^{2+}$ perfusate was completely protective (CAP area recovered to $93 \pm 12\%$ of control; $n = 6$, Fig. 1*A* and *C*). In contrast, bath Ca^{2+} removal was completely ineffective at protecting dorsal columns from 60 min OGD ($7 \pm 9\%$ of control, $n = 8$, Fig. 1*B* and *C versus* $4 \pm 6\%$ in Ca^{2+}-containing perfusate, $P = 1.0$). $0Ca^{2+}$ perfusate alone, without ischaemia, had no significant effect on CAP magnitude after equivalent washout periods (data not shown). Similarly, reducing bath Na^+ to 27 mM by replacing Na^+ with the impermeant cation *N*-methyl-D-glucamine largely rescued dorsal columns from 30 min of OGD (CAP recovery was $82 \pm 25\%$ of control, $n = 4$, Fig. 1*A* and *C*) but was again ineffective against 1 h of OGD ($9 \pm 5\%$ of control, $n = 5$, Fig. 1*B* and *C versus* $4 \pm 6\%$ in normal aCSF, $P = 0.13$). Only depletion of both bath Ca^{2+} and Na^+ was protective, allowing recovery to $66 \pm 9\%$ (Ouardouz *et al.* 2003). Interestingly, selective blockade of voltage-gated Na^+ channels by TTX (0.1 μM) did not protect dorsal columns against either 30 or 60 min OGD (CAP recovery with vs. without TTX, $22 \pm 14\%$ vs. $29 \pm 19\%$ at 30 min ($n = 4$, Fig. 1*A* and *C*, $P = 0.49$); $9 \pm 12\%$ vs. $4 \pm 6\%$ at 1h, ($n = 5$,

Fig. 1*B* and *C*, $P = 1.0$), Exposure to TTX or low Na^+ without OGD for 2 h then 3 h wash in normal CSF allowed CAP areas to recover to $79 \pm 5\%$ ($n = 4$) and $97 \pm 18\%$ ($n = 4$) of control, respectively, indicating only modest depression of excitability by these treatments, which would not account for the failure to protect against OGD. These results indicate that both extracellular Na^+ and Ca^{2+} ions contributed critically to dorsal column injury during the first 30 min of OGD; importantly, longer ischaemia recruited additional injury mechanisms that could not be mitigated by removal of external Ca^{2+} or Na^+. Moreover, our observations also

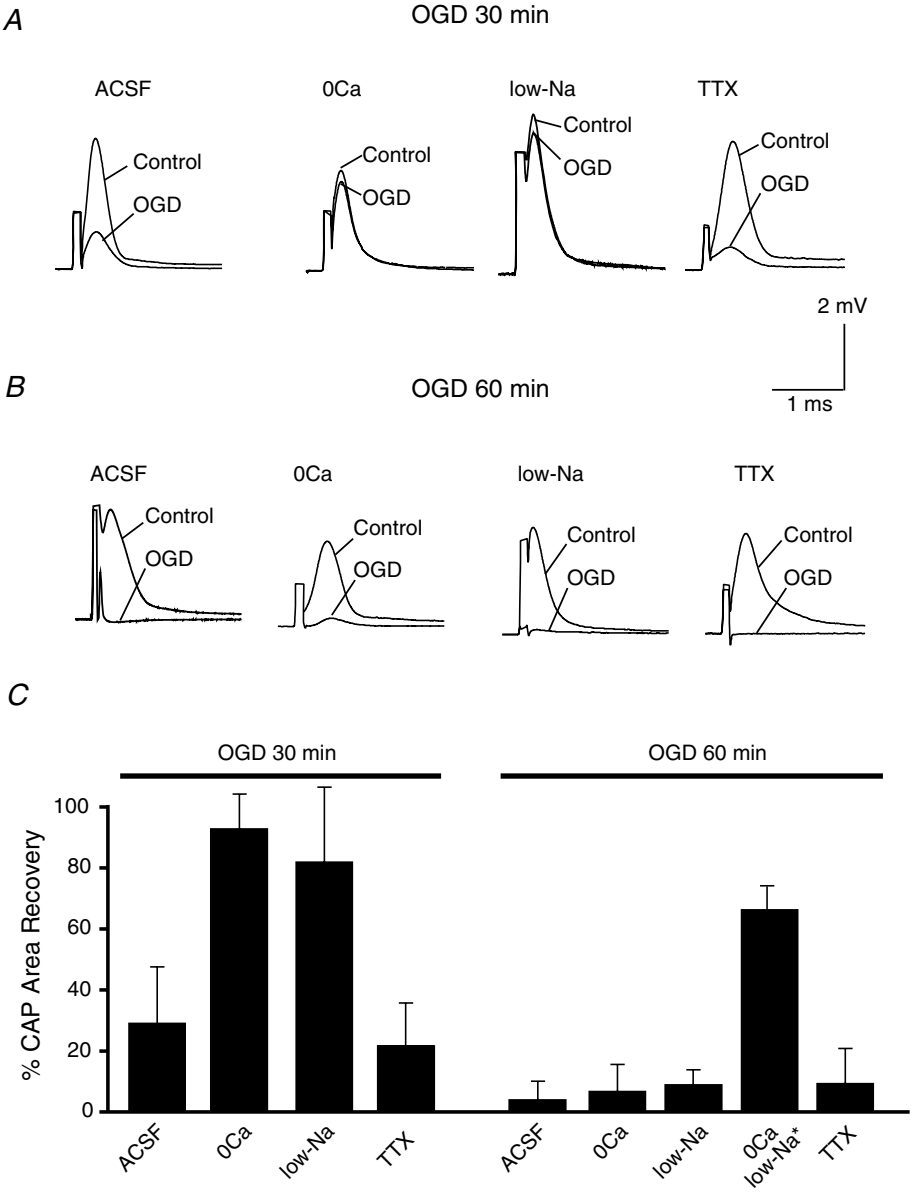

Figure 1. Chemical ischaemia was induced *in vitro* by switching to N_2 atmosphere with glucose replaced by sucrose in the perfusate (oxygen and glucose deprivation, OGD) at 37°C
A, OGD for 30 min, followed by 3 h of reperfusion caused an irreversible reduction of compound action potential (CAP) area which could be rescued by removal of bath Ca^{2+} or by low Na^+ concentration (29 mM) but not by TTX (0.1 μM) blockade of Na^+ channels. *B*, OGD for 60 min caused a severe injury of spinal cord dorsal column as reflected by a near-complete failure of CAP propagation, which could not be prevented by removal of bath Ca^{2+} or Na^+, nor by addition of TTX. *C*, bar graph showing quantitative changes in mean CAP area after 30 or 60 min OGD under different experimental conditions. $n = 8$, 6, 4 and 4 for 30 min OGD for ACSF, 0Ca, low-Na and TTX, respectively, and $n = 12$, 8, 5, 7 and 5 for 60 min OGD for ACSF, 0Ca, low-Na, 0Ca low-Na and TTX, respectively. *Data from Ouardouz *et al.* (2003).

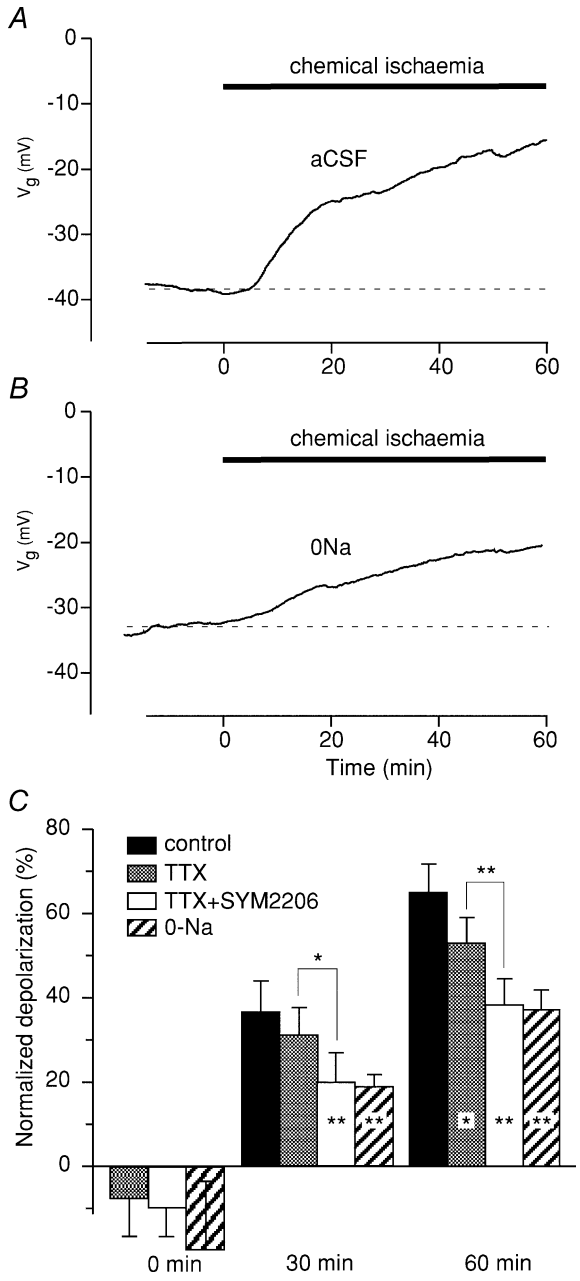

Figure 2. Effect of chemical ischaemia on the compound resting membrane potential of spinal cord dorsal column axons
Time 0 denotes application of insult. Zero percentage depolarization denotes no change from normal resting potential, 100% means complete depolarization to 0 mV. *A*, chemical ischaemia (2 mM NaN_3 + 1 mM iodoacetic acid (IAA)) caused a 65 ± 7% depolarization after 60 min of perfusion. *B*, removal of external Na^+ reduced the degree of depolarization. *C*, summary of different pharmacological manipulations and their effects on ischaemic membrane depolarization. Left group of bars (0 min) shows that all three manipulations caused a significant hyperpolarizing shift in axonal resting potential before ischaemia was applied (see text). Na^+-channel blockade (1 μM TTX) was only modestly effective at preventing depolarization during chemical ischaemia (53 ± 6% depolarization at 60 min *versus* 65 ± 7% without TTX). Combined blockade of Na^+ channels and AMPA receptors (TTX + 30 μM SYM2206) reduced ischaemic depolarization more than TTX alone, to a degree comparable to that observed with Na^+ removal (TTX + SYM2206, 38 ± 6%; 0Na, 37 ± 5%). $^*P < 0.05$, $^{**}P < 0.01$. $n = 5$, 6, 5 and 6 for control, TTX, TTX + SYM2206 and 0Na, respectively.

indicate that TTX-sensitive voltage-gated Na^+ channels are not the only routes by which Na^+ gains access into the intracellular compartment during *in vitro* ischaemia in dorsal columns.

Resting membrane potentials in dorsal column axons

Optic nerve axons exhibit baseline permeability to Na^+ that contributes several millivolts of depolarization at rest (Stys *et al.* 1993; Leppanen & Stys, 1997*a*; Malek *et al.* 2003). Dorsal column fibres also displayed a resting Na^+ permeability: addition of TTX (prior to chemical ischaemia) shifted resting membrane potential by 7 ± 6% ($n = 6$, $P = 0.009$ by paired t test) in the hyperpolarizing direction after 30 min of exposure (Fig. 2*C*). Addition of the AMPA antagonist SYM2206 (30 μM) did not cause any additional hyperpolarization (10 ± 7%, $n = 6$, $P = 0.27$, two-sample t test) indicating that AMPA receptors did not contribute significant ionic permeability in resting axons. During ischaemia, TTX reduced the degree of depolarization (53 ± 6% depolarization at 60 min *versus* 65 ± 7% without TTX, $P = 0.02$). The effect was not significant at 30 min, which was even more restrained with the addition of SYM2206 (SYM2206 + TTX *versus* TTX alone, 20 ± 7% *versus* 31 ± 7%, $P = 0.03$ at 30 min; 38 ± 6% and 53 ± 6%, $P = 0.003$ at 60 min, Fig. 2*C*). The combination of TTX + SYM2206 had a depolarization-sparing effect identical to Na^+-depleted perfusate (38 ± 6% and 37 ± 5% depolarization at 60 min of ischaemia, respectively, $P = 0.99$).

Role of AMPA receptors during OGD

Blockade of AMPA receptors is protective in spinal cord dorsal column anoxia and trauma (Agrawal & Fehlings, 1997; Li *et al.* 1999). The above results suggest that AMPA receptors were activated during ischaemia, but not at rest. Given the known permeability of some AMPA receptors to Na^+ and Ca^{2+} (Hollmann *et al.* 1991), we therefore explored whether glutamate receptor antagonists, in addition to reducing ischaemic depolarization, improve CAP recovery after 1 h OGD (Fig. 3), a protocol where neither Ca^{2+} or Na^+ removal were protective (Fig. 1). Kynurenic acid (1–1.5 mM), a broad-spectrum ionotropic glutamate receptor antagonist, afforded highly significant protection against 1 h OGD (CAP recovery 56 ± 27%, $n = 6$ *versus* 4 ± 6% without antagonist, $P = 1.8 \times 10^{-6}$). Similar results were obtained using the AMPA/kainate receptor antagonist

6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 50 μM; $55 \pm 24\%$ recovery, $n = 6$, $P = 1.8 \times 10^{-6}$). The more selective AMPA receptor inhibitors GYKI52466 (100 μM) and SYM2206 (30 μM) (Ouardouz & Durand, 1991; Donevan & Rogawski, 1993; Pei *et al.* 1999) had similar potency to kynurenic acid and CNQX, with CAP recoveries to $57 \pm 30\%$ ($n = 5$, $P = 1.9 \times 10^{-6}$) and $60 \pm 14\%$ ($n = 3$, $P = 6.6 \times 10^{-6}$) of control, respectively. In addition, blocking glutamate release mediated by reverse Na^+-dependent glutamate transport using dihydrokainate or DL-threo-β-benzyloxyaspartic acid (TBOA, 500 μM) were also highly protective ($49 \pm 28\%$ of control, $n = 6$, $P = 3 \times 10^{-6}$ and $49.1 \pm 19.7\%$ of control $n = 5$, $P = 7 \times 10^{-6}$, respectively), indicating that endogenous glutamate was released by this mechanism during OGD. Taken together, these results indicate that ischaemia-induced glutamate release activated AMPA receptors to cause injury; of note, these receptors did not mediate injury only by fluxing Ca^{2+} or Na^+ ions, because removal of either was not protective in the 1 h OGD protocol.

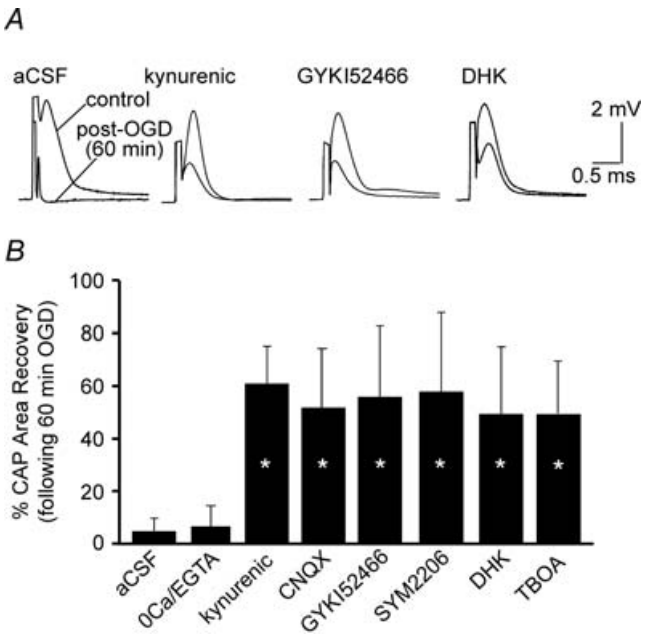

Figure 3. Protective effect of glutamate receptors antagonists
Kynurenic acid (1–1.5 mM), a broad-spectrum glutamate receptor antagonist, CNQX (50 μM, AMPA/kainate receptor antagonist) and the selective AMPA receptor inhibitors GYKI52466 (100 μM) and SYM2206 (30 μM) afforded highly significant protection against 60 min OGD. Blocking glutamate release mediated by reverse Na^+-dependent glutamate transport with dihydrokainate (DHK, 1 mM) or TBOA (500 μM) was also highly protective. *A*, representative CAP tracings. *B*, bar graph showing quantitative changes in mean CAP area recovery after 60 min OGD under different experimental conditions. $^*P < 10^{-4}$ *versus* aCSF alone. $n = 23$, 8, 6, 6, 5, 3, 6, 5 respectively, as shown in bar graph.

Intracellular Ca^{2+} stores during ischaemia

Given the above results, and a report that group 1 mGluR antagonists were protective against dorsal column traumatic injury (Agrawal *et al.* 1998), we examined whether released glutamate may also signal through mGluRs during OGD. Applied by itself, the group 1 mGluR antagonist 1-aminoindan-1,5-dicarboxylic acid (AIDA, 50 μM) afforded no protection against 1 h OGD (CAP recovered to $8 \pm 15\%$ after 1 h OGD/reperfusion, $n = 9$, Fig. 4). However, one cannot *a priori* dismiss any potential contribution of group 1 mGluRs (and by inference inositol

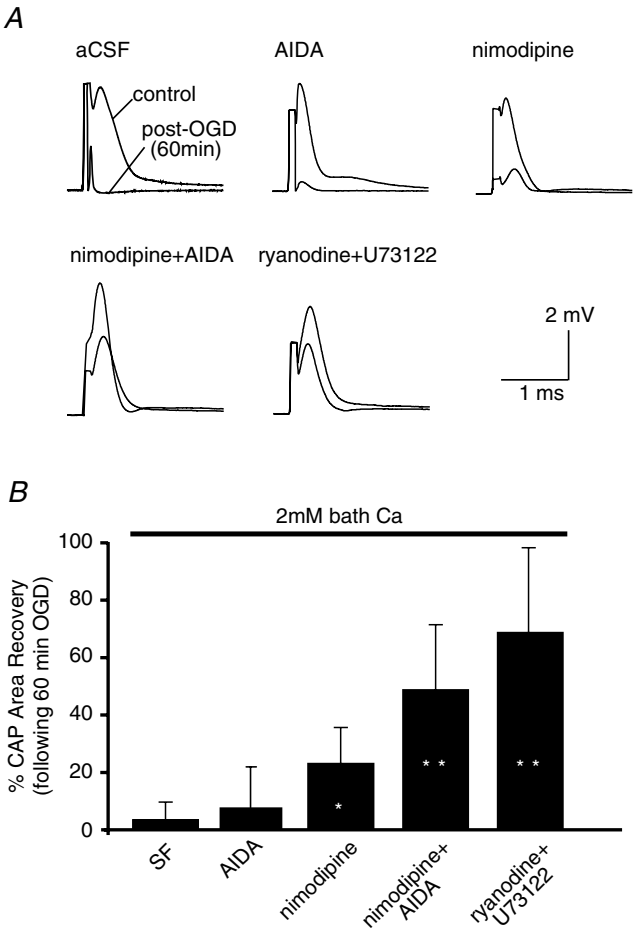

Figure 4. Intracellular Ca^{2+} stores are mobilized during OGD in spinal cord dorsal columns
A, group 1 metabotropic glutamate receptor antagonist AIDA (50 μM) afforded no protection against 60 min OGD in normal external Ca^{2+}. Only the combination of AIDA together with nimodipine (10 μM) was protective. Similarly, when Ca^{2+} release from ryanodine-dependent stores was blocked by ryanodine (50 μM) and IP_3 synthesis reduced by inhibiting phospholipase C (U73122, 20 μM), significant protection was seen. *B*, quantitative summary of CAP area recovery after 3 h reperfusion following 60 min OGD under different experimental conditions. $^*P < 10^{-4}$ *versus* aCSF alone. $n = 23$, 9, 7, 6 and 4 for aCSF, AIDA, nimodipine, nimodipine + AIDA and ryanodine + U73122, respectively, as shown in bar graph.

1,4,5-trisphosphate (IP_3)-dependent Ca^{2+} stores that are typically controlled by these receptors) because CNS white matter injury depends on a number of parallel pathways of Ca^{2+} overload (e.g. Na^+–Ca^{2+} exchanger, L-type Ca^{2+} channels, ryanodine receptors, see Ouardouz *et al.* 2003, 2005). Similarly, the mean recovery in the presence of nimodipine (10 μM) alone was not statistically different from control ($23 \pm 13\%$, $n = 6$, $P = 0.09$). In contrast, the combination of the group 1 mGluR inhibitor AIDA together with nimodipine was protective, with mean CAP recovery of $49 \pm 23\%$ of control, $n = 6$, compared to $4 \pm 6\%$ in aCSF alone, $P = 2.8 \times 10^{-6}$ *versus* aCSF alone (Fig. 4). Similarly, when Ca^{2+} release from ryanodine-dependent stores was blocked by ryanodine (30–50 μM) and IP_3 synthesis reduced by inhibiting phospholipase C (U73122, 20 μM), CAP recovery was also greatly improved ($69 \pm 30\%$, $n = 4$, $P = 1.8 \times 10^{-6}$ *versus* aCSF alone).

These data suggest that intracellular Ca^{2+} sources contributed significantly to dorsal column injury. In order to examine the effects of various manipulations on intracellular Ca^{2+} release, additional studies were performed in perfusate devoid of Ca^{2+} and with the addition of the Ca^{2+} chelator EGTA, to remove any influence of Ca^{2+} entering from the extracellular space. Under these conditions, nimodipine was even more effective than in Ca^{2+}-containing perfusate (CAP area recovered to $57 \pm 22\%$ with $0Ca^{2+}$ + nimodipine, $n = 10$, $P = 2.5 \times 10^{-4}$ *versus* $0Ca^{2+}$ aCSF, Fig. 5*A*). The improved protection with nimodipine observed in $0Ca^{2+}$ aCSF *versus* Ca^{2+}-containing aCSF indicates additional parallel Ca^{2+} influx pathways, such as Na^+–Ca^{2+} exchanger (Ouardouz *et al.* 2005; for review see Stys, 2004). Similarly, in $0Ca^{2+}$ solution, ryanodine or nimodipine alone, or in combination, all conferred roughly the same degree of protection (~55% CAP area recovery, Fig. 5*A*), indicating that under these conditions, nimodipine acted mainly to restrain depolarization-dependent Ca^{2+} release from ryanodine-dependent Ca^{2+} stores (Ouardouz *et al.* 2003). The remaining injury may be due to Ca^{2+} release from ryanodine-independent sources (e.g. IP_3 receptor, mitochondria). Interference with IP_3-dependent Ca^{2+} release at multiple points, including (1) group 1 mGluR inhibition (AIDA), (2) reducing IP_3 synthesis by blocking phospholipase C (U73122) or (3) IP_3 receptor blockade (2-APB) all resulted in nearly identical degrees of postischaemic CAP recovery (~55–60%, Fig. 5*B*). Taken together, these results indicate that IP_3 receptor-dependent Ca^{2+} release also contributed significantly to dorsal column white matter injury. We reasoned that if all three sources of Ca^{2+} were restrained (external influx, ryanodine receptor- and IP_3 receptor-dependent release of intracellular pools) injury should be largely prevented. This was indeed the case: applying nimodipine (to block depolarization-dependent

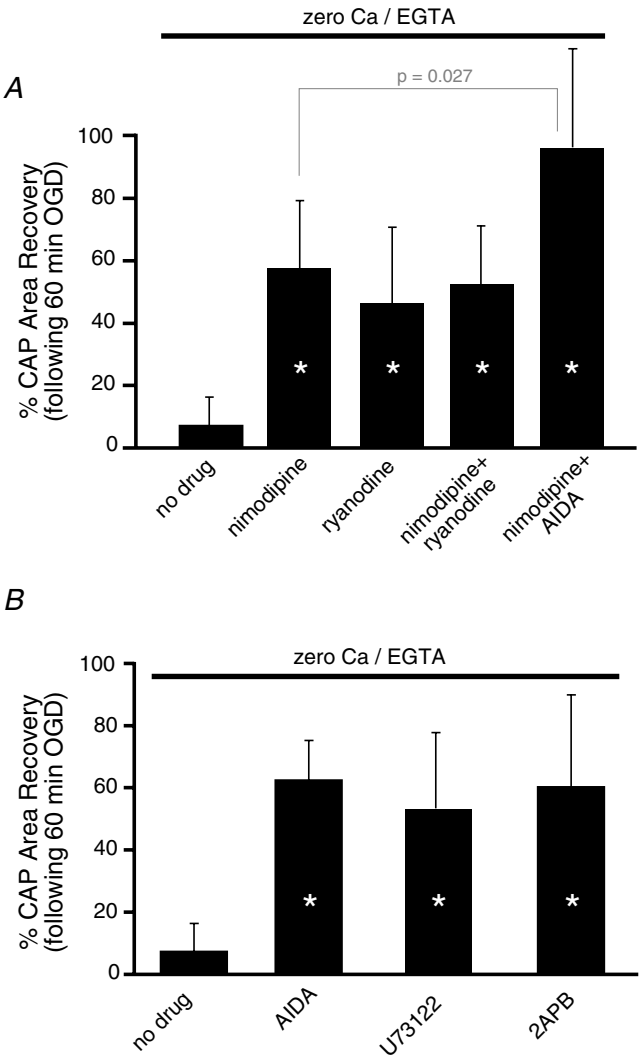

Figure 5. Role of intracellular Ca^{2+} stores during OGD in spinal cord dorsal column

A, quantitative summary of mean CAP area recovery in the presence of various agents after 60 min OGD in the absence of bath Ca^{2+}. Blocking Ca^{2+} release from ryanodine-dependent stores by blocking voltage-gated Ca^{2+} channels (nimodipine, 10 μM) or ryanodine receptors directly (ryanodine, 50 μM) was highly protective, emphasizing the importance of this Ca^{2+} source in ischaemic injury. Eliminating Ca^{2+} influx from the extracellular space while blocking both ryanodine- and IP_3-dependent Ca^{2+} release ($0Ca^{2+}$ + EGTA + nimodipine + 50 μM AIDA) rendered dorsal columns virtually completely resistant to a severe 1 h ischaemic insult. *B*, similarly, interfering with Ca^{2+} release from IP_3-dependent Ca^{2+} stores by blocking group 1 mGluRs (AIDA), inhibiting phospholipase C (and therefore IP_3 synthesis; U73122, 20 μM), or blocking IP_3 receptors directly (2-APB, 100 μM) was also very protective. These results illustrate the three main Ca^{2+} sources in ischaemic spinal axons: extracellular space, ryanodine- and IP_3-dependent Ca^{2+} stores. $n = 8$, 10, 6, 7 and 5 for no drug, nimodipine, ryanodine, nimodipine + ryanodine and nimodipine + AIDA, respectively, as shown in upper bar graph and $n = 8$, 8, 10 and 5 for no drug, AIDA U73122 and 2APB, respectively, as shown in lower bar graph.

release of ryanodine-dependent stores; Ouardouz *et al.*2003) and AIDA (to block signalling that converges onto IP_3-dependent Ca^{2+} stores) in the absence of external Ca^{2+}, completely rescued dorsal columns from 60 min of OGD (CAP area recovery to $97 \pm 32\%$ of control, $n = 5$, *versus* $4 \pm 6\%$ in Ca^{2+}-containing aCSF with no blockers, $P < 10^{-5}$ *versus* 0 Ca^{2+}; $P = 0.027$ *versus* $0Ca^{2+}$ + nimodipine, Fig. 5*A*).

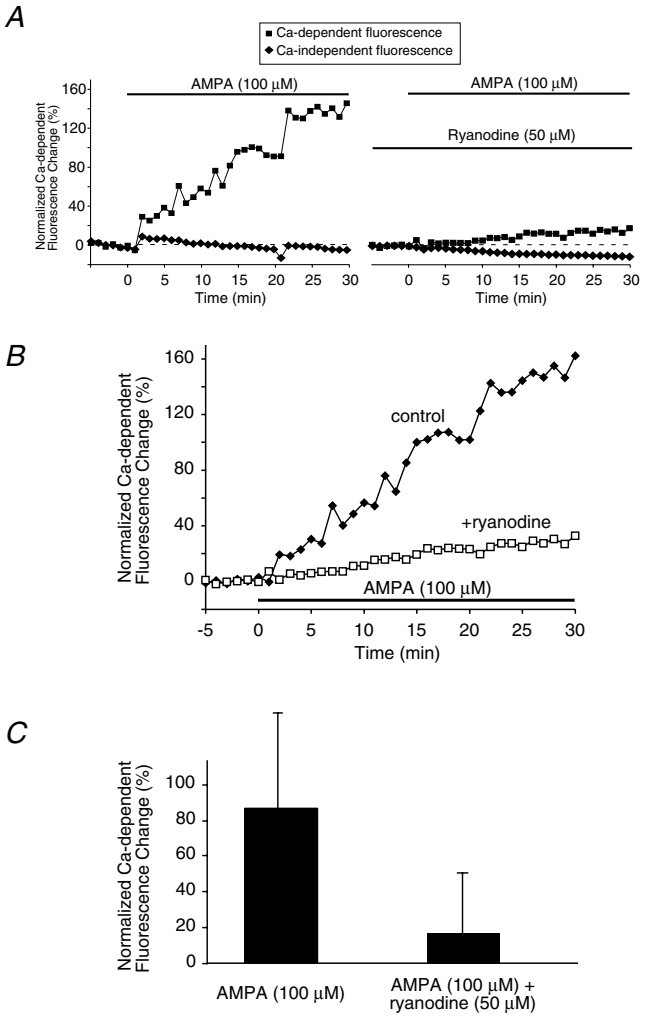

Figure 6. AMPA increases axoplasmic [Ca^{2+}] in spinal cord dorsal column axons measured using confocal microscopy
A and *B*, example time courses from one experiment of axoplasmic Ca^{2+} levels as reported by fluorescence change of Oregon Green-488 BAPTA-1 in normal, non-ischaemic dorsal column axons. AMPA (100 μM; plus 30 μM cyclothiazide to reduce desensitization) induced a substantial Ca^{2+} rise which was significantly blunted by ryanodine. *A*, shows Ca^{2+}-dependent (Oregon Green-488 BAPTA-1) and Ca^{2+}-independent (Alexa Fluor 594) fluorescence separately. *B*, illustrates the ratio of Ca^{2+}-dependent to Ca^{2+}-independent fluorescence to correct for the small downward drift in fluorescence over time. *C*, bar graph of mean axoplasmic Ca^{2+} fluorescence changes at 30 min. Fluorescence increased by a mean of $86 \pm 53\%$ above baseline after 30 min of AMPA exposure. Addition of ryanodine (50 μM) reduced the AMPA-evoked Ca^{2+} increase to $16 \pm 34\%$ ($P < 10^{-7}$). These data suggest that AMPA receptors increase axonal [Ca^{2+}] partly by controlling release of Ca^{2+} from ryanodine receptor-dependent axonal Ca^{2+} stores (see text). $n = 48$ and 26 axons for AMPA and AMPA + ryanodine, respectively.

AMPA promotes axoplasmic Ca^{2+} rise partly from ryanodine-dependent Ca^{2+} stores

Taken together, the results thus far strongly suggest a mechanistic link between AMPA receptors and intra-axonal Ca^{2+} stores. To directly demonstrate such a connection, we activated AMPA receptors selectively in uninjured, non-ischaemic dorsal column slices using AMPA (100 μM, plus 30 μM cyclothiazide to reduce receptor desensitization) while imaging axoplasmic Ca^{2+} changes using Oregon Green-488 BAPTA-1 fluorescence (Fig. 6). After 30 min of exposure, fluorescence increased by $86 \pm 53\%$ over baseline ($n = 48$ axons from seven different experiments). Blocking ryanodine receptors (50 μM ryanodine) significantly reduced (but did not completely abolish) the AMPA-evoked axonal Ca^{2+} rise ($16 \pm 34\%$ at 30 min, $n = 26$ axons from three experiments, $P = 10^{-7}$). In order to help exclude a potential glial contribution to the effects of AMPA on axonal Ca^{2+} rise, myelinic Ca^{2+} was measured in response to application of AMPA + cyclothiazide. After 30 min of exposure, Ca^{2+}-dependent fluorescence did not change significantly ($-4 \pm 5\%$, $n = 28$ myelin regions).

Axoplasmic Ca^{2+} changes during chemical ischaemia

Using confocal microscopy, relative axoplasmic [Ca^{2+}] was then measured during chemical ischaemia at 10-min intervals for 60 min. Representative micrographs are shown in Fig. 7*A*. In Ca^{2+}-containing perfusate, axoplasmic Ca^{2+}-dependent fluorescence gradually increased to $99 \pm 50\%$ above pre-ischaemic baseline after 30 min and $198 \pm 68\%$ after 60 min ischaemia ($n = 30$ axons). In the absence of external Ca^{2+} (with the addition of 0.5 mM EGTA which caused the extracellular Ca^{2+} to fall to negligible levels within 4 min; Nikolaeva *et al.* 2005) ischaemia still induced a rise of axoplasmic [Ca^{2+}], though to a lesser degree ($54 \pm 54\%$ in $0Ca^{2+}$ compared to $99 \pm 50\%$ in normal Ca^{2+} after 30 min, $P = 10^{-4}$ *versus* Ca^{2+}-containing aCSF, and $116 \pm 68\%$ in $0Ca^{2+}$ compared to $198 \pm 68\%$ in normal Ca^{2+} after 60 min, $P = 7 \times 10^{-5}$, $n = 32$ and 30 axons, respectively). These results confirm that significant amounts of Ca^{2+} are sourced from intracellular compartments during injury in these axons (Ouardouz *et al.* 2003). Combined application of AIDA and nimodipine in the absence of external Ca^{2+} almost completely blocked Ca^{2+} increase after 30 min ($5 \pm 16\%$ *versus* $54 \pm 54\%$ in $0Ca^{2+}$ + EGTA, $P = 10^{-5}$, $n = 45$ and 32 axons, respectively). This block

was less pronounced but still significant after 60 min of ischaemia ($67 \pm 60\%$ *versus* $116 \pm 68\%$, $P = 0.04$). The AMPA antagonist SYM2206 (30 μM) also significantly reduced ischaemia-induced axonal increase in Ca^{2+} concentration at 30 min ($28 \pm 9\%$ *versus* $99 \pm 50\%$ in normal Ca^{2+}-containing aCSF, $P = 2.4 \times 10^{-5}$) and 60 min ($86 \pm 37\%$ *versus* $198 \pm 68\%$, $P = 3.7 \times 10^{-4}$).

Immunolocalization of AMPA receptors

Dorsal columns were immunostained with antibodies specific for GluR1, GluR2/3 and GluR4, the main subunits making up AMPA receptors (Fig. 8). Axon cylinders were labelled by neurofilament antibody which is shown as red in Fig. 8. GluR1 staining was patchy and mostly appeared just outside larger axons, in regions of the myelin sheath. GluR2/3 (probably GluR3 only, see legend to Fig. 8) and GluR4 signals on the other hand were punctate, and were localized at the edges of neurofilament-positive axonal profiles, suggesting expression on the inner myelin loops and/or the axolemma.

Discussion

Acute energy failure induced by OGD caused irreversible damage to spinal cord dorsal columns. In contrast to the situation with anoxia (Imaizumi *et al.* 1997) or trauma (Agrawal & Fehlings, 1996), here we show that inhibition of voltage-gated Na^+ channels by TTX or removal of external Na^+ was not protective against 60 min of OGD. This

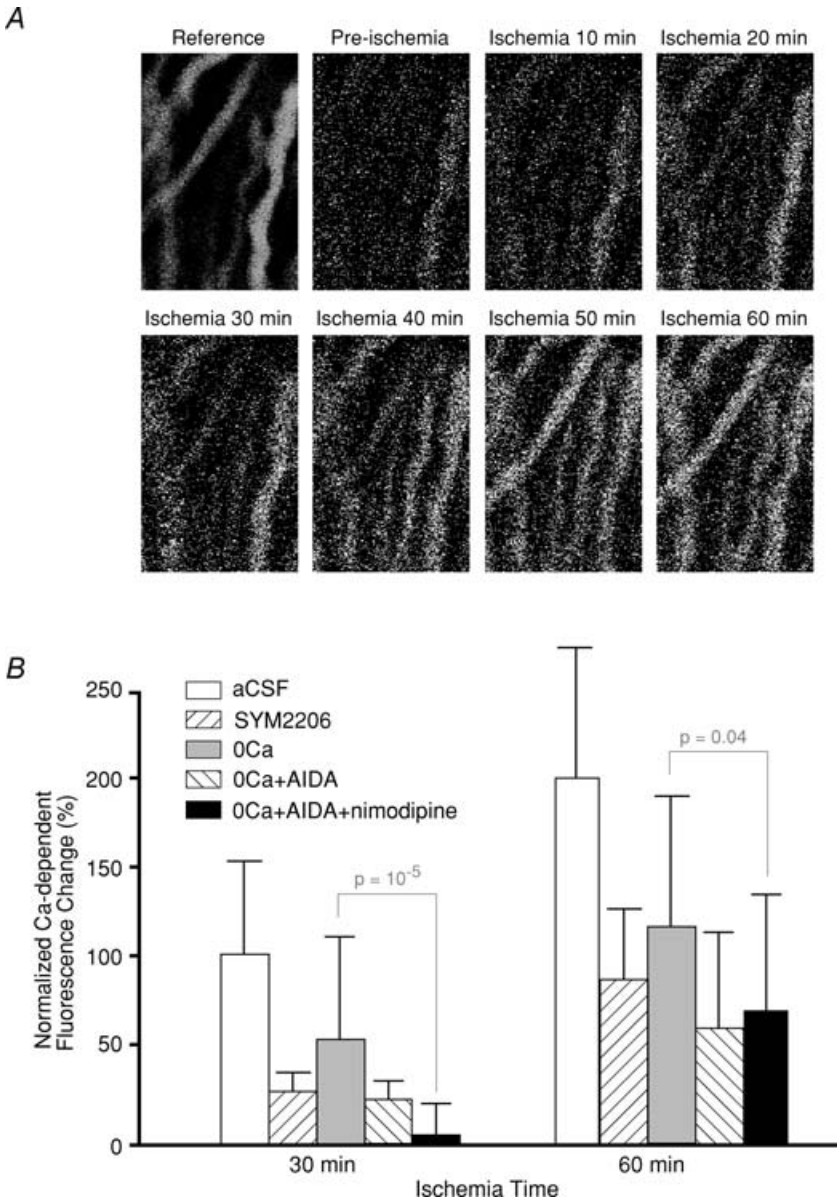

Figure 7. Confocal imaging of axoplasmic $[Ca^{2+}]$ in spinal cord dorsal column axons during chemical ischaemia
A, representative micrographs showing axon profiles outlined by a red Ca^{2+}-insensitive dextran-conjugated fluorophore (Reference). Remaining panels show Ca^{2+}-sensitive emission in pseudocolour from pre-ischaemic fibres and at 10-min intervals during ischaemia (induced using NaN_3 + iodoacetic acid (IAA)). *B*, bar graph showing quantitative changes in axoplasmic Ca^{2+}-dependent fluorescence. Ischaemia increased axoplasmic Ca^{2+} levels in Ca^{2+}-containing perfusate, which was reduced but not prevented by removing bath Ca^{2+} (with the addition of 0.5 mM EGTA). Addition of AIDA (50 μM) to $0Ca^{2+}$ + EGTA perfusate blunted the ischaemic $[Ca^{2+}]$ increase (not significant with respect to $0Ca^{2+}$ alone). However, combined application of AIDA and nimodipine (10 μM) in the absence of external Ca^{2+} significantly blocked axonal $[Ca^{2+}]$ rise. The AMPA antagonist SYM2206 (30 μM) was highly effective even in the presence of bath Ca^{2+}. $n = 30$, 9, 32, 16 and 45 for aCSF, SYM2206, 0Ca, $0Ca^{2+}$ + AIDA, $0Ca^{2+}$ + AIDA + nimodipine.

discrepancy is likely to be due to the difference in severity of the insults. It is interesting that 0Na^+ solution rescued spinal cord dorsal columns from a shorter (30 min) OGD exposure whereas TTX was ineffective (Fig. 1). This indicates that shorter ischaemic injury is heavily dependent on extracellular Na^+ ions, but that Na^+ influx pathways other than TTX-sensitive Na^+ channels play an important role. These results underscore the relatively minor role of voltage-gated Na^+ channels in dorsal column ischaemia compared to other CNS white matter tracts such as optic nerve (see Fig. 7 in Stys, 2004), even though dorsal column axons appear to exhibit a resting non-inactivating permeability to Na^+ that is similar in magnitude to that in optic nerve (Fig. 2*B*; Leppanen & Stys, 1997*a*). Similarly, removal of extracellular Ca^{2+} protected against 30 min, but was ineffective after 60 min of OGD; removal of both Na^+ and Ca^{2+} ions from the bath was necessary to protect dorsal columns from longer OGD exposures (Ouardouz *et al.* 2003). Analysis of resting membrane potentials during ischaemia indicates that voltage-gated Na^+ channels are not the sole mediators of depolarization: in contrast to the resting state, where AMPA receptors appear not to contribute to resting membrane potential, during ischaemia these receptors become activated and contribute to ischaemic depolarization as much if not more than Na^+ channels (Fig. 2*C*). Indeed, adding AMPA receptor blockade to TTX significantly reduced the degree of ischaemic axonal depolarization at 30 and 60 min (Fig. 2*C*), indicating that AMPA receptors, in addition to compromising glia, may represent an important route for Na^+ influx into ischaemic dorsal column axons. Moreover, the degree of depolarization at 30 and 60 min of ischaemia in the presence of TTX + SYM2206 was identical to that observed in 0Na^+ perfusate, suggesting that together, Na^+ channels and AMPA receptors may represent the two major avenues of Na^+ flux that in turn promote axonal depolarization in ischaemic dorsal columns.

The data above indicate that as the injury intensifies, an increasingly complex interplay of damaging Na^+- and Ca^{2+}-dependent pathways occurs. In light of this, it is remarkable that AMPA receptor inhibitors were so protective against prolonged (60 min) OGD even in Ca^{2+}-containing perfusate, a protocol where total Ca^{2+} or Na^+ removal was completely ineffective. Although GYKI52466 and SYM2206 have been extensively used as selective AMPA receptor antagonists (Ouardouz & Durand, 1991; Donevan & Rogawski, 1993; Pei *et al.* 1999), we found that both agents also blocked currents carried

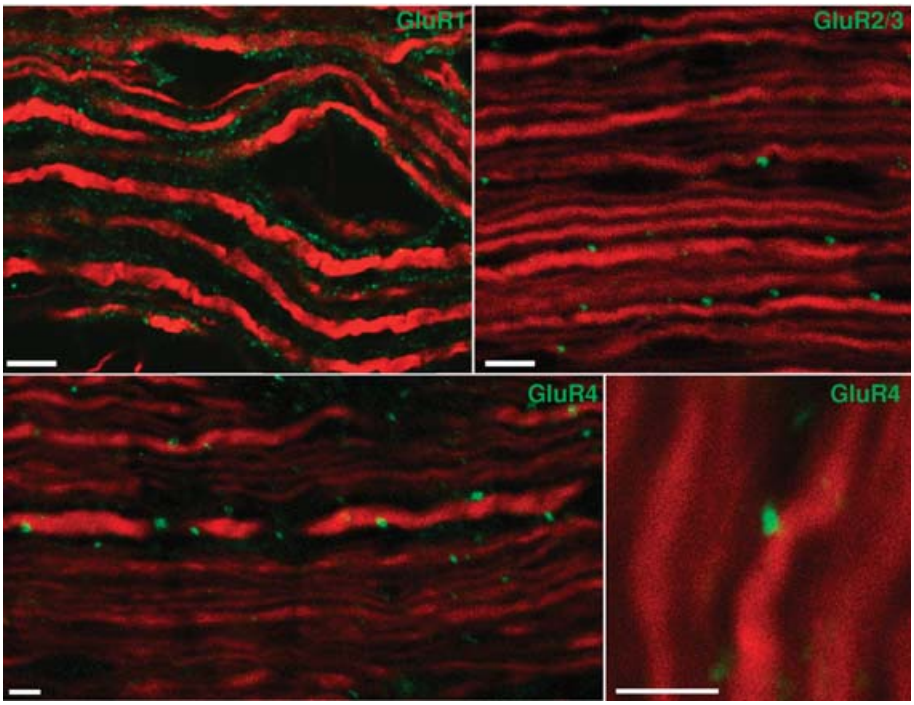

Figure 8. Rat dorsal column sections were immunostained with antibodies recognizing the GluR1–4 AMPA receptor subunits (green)
Axon cylinders are identified by neurofilament (red). GluR1 staining was patchy but was seen throughout the thickness of the myelin sheath. In contrast, GluR2/3 and GluR4 staining was punctate, and was localized at the edges of neurofilament-positive axonal profiles. Given the absence of GluR2 subunits in rat spinal white matter shown previously (Park *et al.* 2003), our GluR2/3 label most probably reflects the presence of GluR3 subunits. The limited resolution of light microscopy does not permit us to conclusively localize the GluR3 and 4 clusters to inner myelin loops *versus* axolemma. Scale bars, 5 μm.

by $Na_v1.6$ at concentrations routinely used to inhibit AMPA receptors (C. E. Morris, W. Lin and P. K. Stys, unpublished observations). The protective effect obtained by GYKI52466 and SYM2206 was similar to kynurenic acid and CNQX. Therefore, the actions of GYKI52466 and SYM2206 in this study are ascribable mainly to AMPA receptor-dependent mechanisms. In addition, TTX and removal of external Na^+ were completely ineffective; therefore, it is unlikely that the neuroprotective effects of GYKI52466 and SYM2206 were due to $Na_v1.6$ block. Taken together, our data are consistent with the notion that during prolonged dorsal column ischaemia, transaxolemmal Na^+ and Ca^{2+} flux, and secondary release of Ca^{2+} from Ca^{2+} stores (Ouardouz *et al.* 2003), appear to be governed by AMPA receptors to a significant degree. Moreover, the glutamate transport blocker dihydrokainate had similar effects, demonstrating that this pathway can be blocked either at the receptor end, or at the point of transmitter release (Li *et al.* 1999). Even then, there remained ∼40% electrophysiological injury that is under the control of distinct signalling pathways (see below).

AMPA receptor activation has been reported to be involved in white matter injury (Agrawal & Fehlings, 1997; Wrathall *et al.* 1997; Li *et al.* 1999; Rosenberg *et al.* 1999; Tekkok & Goldberg, 2001). Whereas the precise mechanisms have not been fully elucidated, the presence of these receptors on both astrocytes and oligodendrocytes (Li & Stys, 2000; Verkhratsky & Steinhauser, 2000), and their ability to damage these cells (Oka *et al.* 1993; David *et al.* 1996; Matute *et al.* 1997; McDonald *et al.* 1998; Chen *et al.* 2000; Li & Stys, 2000), suggests that at least part of the injury observed in traumatic, anoxic and ischaemic spinal white matter may be due to disruption of glial elements. Studies in white matter from different central nervous system regions have shown AMPA/kainate receptors in oligodendrocytes, astrocytes and axons (Matute & Miledi, 1993; Patneau *et al.* 1994; Garcia-Barcina & Matute, 1996; Agrawal & Fehlings, 1997; Li & Stys, 2000; Brand-Schieber & Werner, 2003). Accordingly, one explanation for the axonal protective effect of AMPA receptor antagonists was suggested to be secondary to sparing of glial elements, leading to a reduction of diffusible messengers (e.g. NO and free radicals) (for review see Matute *et al.* 2001). However, exposure of dorsal columns to kainate (an AMPA and kainate receptor agonist) induces axonal rises in $[Ca^{2+}]$; addition of extracellular scavengers of NO and other free radicals does not abolish the kainate-induced $[Ca^{2+}]$ increase (Ouardouz & Stys, 2004). Moreover, the highly localized expression of clusters of GluR3 and GluR4 immediately adjacent to neurofilament profiles (Fig. 8), together with the observed membrane potential measurements (which reflect purely axonal, and not glial potentials), the AMPA-inducible release of Ca^{2+} from axonal ryanodine-dependent stores, and the lack of myelinic Ca^{2+} increase in response to AMPA receptor activation, suggest that in addition to a potential action on glial cells that cannot be excluded, AMPA receptors may directly mediate injury to axons.

The present data confirm our previous findings of ryanodine receptor-dependent ischaemic injury (Ouardouz *et al.* 2003) and further extend this finding by implicating IP_3 receptor-dependent Ca^{2+} stores; interfering with this pathway at three separate points (group 1 mGluR block with AIDA, inhibition of phospholipases C with U73122 or IP_3 receptor block with 2APB) was highly protective in $0Ca^{2+}$ perfusate (Fig. 5*B*). Indeed, removing external Ca^{2+} and restraining both ryanodine- and IP_3-dependent stores in combination ($0Ca^{2+}$ + nimodipine + AIDA, Fig. 5*A*) was completely protective against a very severe 1 h ischaemic insult; this identifies the three most important sources of noxious Ca^{2+} mobilized by this protocol. The second surprising finding was the potent protective effect of AMPA receptor antagonists vis-à-vis the ineffectiveness of bath Ca^{2+} removal. This observation, together with the injurious effects of intracellular Ca^{2+} release, and ryanodine-blockable axonal Ca^{2+} increase induced by AMPA (Fig. 6), point to an intimate link between AMPA receptor signalling and axonal Ca^{2+} stores. In addition to admitting Ca^{2+} from the extracellular space, AMPA receptors may control internal Ca^{2+} pools by two potential mechanisms. The first may involve flux of Na^+ into axons which stimulates release of Ca^{2+} from ryanodine- and IP_3-dependent Ca^{2+} stores in optic nerve (Nikolaeva *et al.* 2005). Indeed, given that TTX-sensitive Na^+ channels contribute to resting axonal membrane potential, whereas AMPA receptors do not (Fig. 2*C*), it is conceivable that the former initiate axonal Na^+ loading and depolarization early in ischaemia, triggering initial glutamate release leading to activation of AMPA receptors. These then assume a dominant role in Na^+ influx, causing axonal depolarization and more glutamate release, thus establishing a positive feedback loop. The other intriguing possibility is that the traditionally ionotropic AMPA receptors signal in a 'non-canonical' manner, by exerting a modulatory influence rather than fluxing ions. Metabotropic actions have been recently reported for both AMPA (Satake *et al.* 2004; Takago *et al.* 2005) and kainate receptors (Melyan *et al.* 2002; Rozas *et al.* 2003) mediated in part via a G-protein, phospholipase C-dependent pathway (Rodriguez-Moreno & Lerma, 1998). Our results using the phospholipase C inhibitor U73122 may in fact reflect such a mechanism, stimulated by metabotropic behaviour of AMPA receptors. Additional studies are required to definitively prove a non-ionotropic action of AMPA receptors in spinal white matter. It is also highly likely that such a runaway release of axoplasmic glutamate (Li *et al.* 1999) also activates NMDA receptors on adjacent adaxonal myelin (Micu *et al.* 2006) and neighbouring

oligodendrocytes (Karadottir *et al.* 2005; Salter & Fern, 2005), promoting damage to these elements.

Confocal microscopy revealed a sustained increase of intra-axonal [Ca^{2+}] during 1 h of *in vitro* ischaemia; removal of extracellular Ca^{2+} reduced but did not eliminate the intra-axonal Ca^{2+} increase, which was previously shown to be sufficient to irreversibly damage this tissue (Ouardouz *et al.* 2003, 2005). One of the mechanisms by which Ca^{2+} is released from stores in dorsal column axons involves activation of ryanodine receptors by axonal L-type Ca^{2+} channels gated by ischaemic depolarization (Ouardouz *et al.* 2003). A second major intracellular Ca^{2+} pool is controlled by IP_3 receptors. Activation of phospholipase C by certain G-protein-coupled metabotropic receptors (Pin & Duvoisin, 1995; Recasens & Vignes, 1995), including type 1 metabotropic glutamate receptors, induces formation of IP_3 and diacylglycerol (Bockaert *et al.* 1993). Our electrophysiological and Ca^{2+} imaging experiments are consistent with the idea of multiple Ca^{2+} sources as described above. Our results also emphasize that additional deleterious pathways are recruited as the insult grows more severe, and restraining any one or even several is necessary but may not be sufficient to confer overall functional protection. Indeed, even blocking multiple pathways of Ca^{2+} accumulation still allowed a substantial late rise in [Ca^{2+}] (Fig. 7), suggesting yet additional undefined axonal Ca^{2+} sources recruited by longer insults. Finally, our data strongly support a central role of AMPA receptor activation during dorsal column ischaemia, which leads to functional injury and a deleterious axoplasmic rise in [Ca^{2+}]; inhibition of these receptors confers potent neuroprotection in central white matter.

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Acknowledgements

This work was supported by National Institute of Neurological Disorders and Stroke (NINDS) R01 NS040087-06; operating grant no. MOP-37858 and equipment grant no. MMA-48299 from the Canadian Institutes of Health Research. P.K.S. was supported by a Heart and Stroke Foundation of Ontario Career Investigator Award.