Glutamate Receptors on Myelinated Spinal Cord Axons: I. GluR6 Kainate Receptors

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Objective: The deleterious effects of glutamate excitotoxicity are well described for central nervous system gray matter. Although overactivation of glutamate receptors also contributes to axonal injury, the mechanisms are poorly understood. Our goal was to elucidate the mechanisms of kainate receptor-dependent axonal Ca^{2+} deregulation.

Methods: Dorsal column axons were loaded with a Ca^{2+} indicator and imaged in vitro using confocal laser-scanning microscopy.

Results: Activation of glutamate receptor 6 (GluR6) kainate receptors promoted a substantial increase in axonal $[\text{Ca}^{2+}]$. This Ca^{2+} accumulation was due not only to influx from the extracellular space, but a significant component originated from ryanodine-dependent intracellular stores, which, in turn, depended on activation of L-type Ca^{2+} channels: ryanodine, nimodipine, or nifedipine blocked the agonist-induced Ca^{2+} increase. Also, GluR6 stimulation induced intraaxonal production of nitric oxide (NO), which greatly enhanced the Ca^{2+} response: quenching of NO with intraaxonal (but not extracellular) scavengers, or inhibition of neuronal NO synthase with intraaxonal N ω -nitro-L-arginine methyl ester, blocked the Ca^{2+} increase. Loading axons with a peptide that mimics the C-terminal PDZ binding sequence of GluR6, thus interfering with the coupling of GluR6 to downstream effectors, greatly reduced the agonist-induced axonal Ca^{2+} increase. Immunohistochemistry showed GluR6/7 clusters on the axolemma colocalized with neuronal NO synthase and $\text{Ca}_v1.2$.

Interpretation: Myelinated spinal axons express functional GluR6-containing kainate receptors, forming part of novel signaling complexes reminiscent of postsynaptic membranes of glutamatergic synapses. The ability of such axonal "nanocomplexes" to release toxic amounts of Ca^{2+} may represent a key mechanism of axonal degeneration in disorders such as multiple sclerosis where abnormal accumulation of glutamate and NO are known to occur.

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Glutamate is the main excitatory neurotransmitter in the mammalian central nervous system, playing a significant role in gray matter injury in many neurodegenerative diseases.¹ Prevalent and devastating disorders such as stroke, multiple sclerosis, and trauma to the brain and spinal cord invariably affect afferent and efferent white matter tracts, though much less is known about mechanisms of injury to myelinated white matter axons. Voltage-gated Na^+ and Ca^{2+} channels, together with reverse Na^+ - Ca^{2+} exchange, play important roles^{2–4} (for review, see Stys⁵). Perhaps counterintuitive, given the nonsynaptic nature of central nervous system white matter, are observations of functional protection of this tissue by antagonists of ionotropic

glutamate receptors. α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)/kainate receptor antagonists are protective both in vitro^{6–10} and in vivo,^{11–14} in ischemic, traumatic, and autoimmune models of white matter injury. Conversely, activating AMPA/kainate receptors, but not *N*-methyl-D-aspartate (NMDA) receptors, or increasing extracellular glutamate levels by blocking glutamate transport either in vitro^{15–17} or in vivo^{17–19} is injurious to axons.

The precise mechanisms of injury to white matter elements induced by non-NMDA glutamate receptor activation are unknown. Both astrocytes and oligodendrocytes express AMPA and kainate receptors (for review, see Matute and colleagues²⁰), and more recently,

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NMDA receptors have been detected on mature oligodendrocytes,²¹ their processes,²² and even the myelin sheath.²³ These receptors are permeable to Ca^{2+} ions; therefore, it is reasonable to conclude that receptor-mediated Ca^{2+} overload is responsible for excitotoxic glial injury.^{15,24,25} What is so far unexplained is the observation that central axons per se are damaged by activation of AMPA/kainate receptors^{18,19} and, in turn, protected by blockers of these receptors in various injury models.^{9,13,26} These latter observations raise the possibility that central myelinated axons themselves express AMPA/kainate receptors, whose overactivation results in damage to the fibers directly. Indeed, antagonists of AMPA/kainate receptors, but not NMDA receptors, were protective against spinal cord dorsal column injury,^{6–8} and bath application of AMPA, kainate, or glutamate, but not NMDA, induced irreversible reduction of compound action potential.^{6,16} In this report, we tested the hypothesis that myelinated axons from rat spinal cord express functional kainate receptors capable of mediating a potentially deleterious axonal Ca^{2+} increase. We found that GluR6-containing kainate receptors reside along the internodal axolemma in “nanocomplexes” together with neuronal nitric oxide synthase (nNOS), exerting control over L-type Ca^{2+} channels and causing Ca^{2+} release from intraaxonal Ca^{2+} stores. These signaling molecules are organized in a surprisingly intricate arrangement (see Fig 6) reminiscent of what is found at the postsynaptic membrane of conventional glutamatergic synapses.

Materials and Methods

All experiments were performed in accordance with institutional guidelines for the care and use of experimental animals. Additional details can be found in the supplementary material.

Ca^{2+} Imaging

Dorsal columns from deeply anesthetized adult Long-Evans male rats were removed from the thoracic region and placed in cold, oxygenated zero- Ca^{2+} solution (containing in mM: NaCl 126, KCl 3, MgSO_4 2, NaHCO_3 26, NaH_2PO_4 1.25, MgCl_2 2, dextrose 10 and EGTA 0.5, oxygenated with 95% O_2 /5% CO_2), loaded for 2 hours with Ca^{2+} -insensitive reference dye (red dextran-conjugated Alexa 594, 250 μM) to allow identification of axon profiles (Fig 1A), together with the dextran-conjugated Ca^{2+} indicator Oregon Green BAPTA-1 (250 μM), and imaged on a Nikon C1 (Toronto, Ontario) confocal microscope at 37°C. All reported axonal $[\text{Ca}^{2+}]$ changes ($F_{\text{Ca,ax}}$) are ratios of green to red fluorescence after 30 minutes of drug application.

Immunohistochemistry and Immunoelectron Microscopy

Immunohistochemistry, immunoelectron microscopy, and immunohistochemistry were performed using standard techniques²³ (see supplemental material).

Peptide Synthesis and Purification

Two peptides (NH_2 -Cys-Ahx-Arg-Leu-Pro-Gly-Lys-Glu-Thr-Met-Ala-CONH₂ (I), [molecular weight = 1,218] and NH_2 -Cys-Ahx-Cys-Ahx-Cys-Ahx-Cys-Ahx-Arg-Leu-Pro-Gly-Lys-Glu-Thr-Met-Ala-CONH₂ (II) [molecular weight = 1,864]) were designed that contain the C-terminal of GluR6 PDZ1 binding motif, a single or multiple N-terminal Cys residues (for dye conjugation via free SH groups), and one or more Ahx (ϵ -amino-hexanoic acid) moieties as spacers (for steric reasons). Active and sham dextropeptides were synthesized using standard methods. The peptides were dissolved to a concentration of 0.1 to 1 mM in the loading pipette yielding approximately 1 to 10 μM in the axons.

Results

Activation of GluR6-Containing Receptors Increases Axonal Ca^{2+}

We measured $[\text{Ca}^{2+}]$ changes in live adult rat dorsal column axons in vitro using laser-scanning confocal microscopy (see Fig 1). Activation of kainate receptors (kainate 200 μM), at concentrations that significantly reduced compound action potentials (see later), caused a progressive increase of intraaxonal $[\text{Ca}^{2+}]$. Axoplasmic Ca^{2+} -dependent fluorescence ($F_{\text{Ca,ax}}$) showed a robust increase after drug application (mean increase after 30 minutes: kainate, $110 \pm 67\%$; $n = 54$ axons) that was strongly reduced by the AMPA/kainate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX 50 μM) ($12 \pm 15\%$; $n = 35$; $p \approx 0$). The AMPA receptor antagonists 1-naphthyl acetyl spermine (25 μM) or 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine (GYKI52466 100 μM) did not significantly blunt kainate-induced $F_{\text{Ca,ax}}$ increase (kainate + spermine: $97 \pm 64\%$, $n = 54$, $p = 0.98$; kainate + GYKI52466: $79 \pm 65\%$, $n = 40$, $p = 0.24$). In contrast, 3-(hydroxyamino)-6-nitro-6,7,8,9-tetrahydrobenzo[g]indol-2-one (NS-102 10 μM), an antagonist of GluR6-containing kainate receptors,²⁷ strongly reduced the response induced by kainate (kainate + NS-102: $35 \pm 25\%$; $n = 37$; $p \approx 0$). (S)-1-(2-amino-2-carboxyethyl)-3-(2-carboxybenzyl)pyrimidine-2,4-dione (UPB-302, 20 μM), a blocker of GluR5-containing kainate receptors,²⁸ was less effective (kainate + UPB-302: $74 \pm 40\%$; $n = 36$) than CNQX or NS-102 at blocking the kainate-induced Ca^{2+} responses ($p \leq 0.012$, kainate + UPB-302 vs kainate + CNQX or kainate + NS-102) (see Fig 1C), indicating that kainate mainly (but not exclusively) activated kainate receptors containing GluR6 subunits. (2S,4R)-4-methyl glutamic acid (SYM2081; 100 μM), another kainate receptor agonist,²⁹ induced an increase of $F_{\text{Ca,ax}}$ ($135 \pm 67\%$; $n = 79$) with a similar pharmacological profile to kainate: The Ca^{2+} response was reduced by CNQX (SYM2081 + CNQX: $38 \pm 24\%$; $n = 29$; $p = 4 \times 10^{-10}$) and NS102 ($25 \pm 35\%$; $n = 28$; $p = 4 \times 10^{-10}$), and also was modestly reduced by

Fig 1. $[\text{Ca}^{2+}]$ in dorsal column axons in response to kainate receptor activation. (A) Confocal micrographs of axons loaded with red dextran-conjugated reference dye together with the Ca^{2+} indicator Oregon Green-488 BAPTA-1 shown in pseudocolor. Activating kainate receptors with kainate induced an increase in Ca^{2+} -dependent fluorescence in the axoplasm. (B) Representative time course of axonal Ca^{2+} increase in response to bath application of agonist at time zero (arrow). Black diamonds represent green/red ratio; gray triangles represent Ca fluorescence; and gray squares represent reference fluorescence. (C) Bar graph showing mean percentage change ($\pm$ standard deviation) in axonal Ca^{2+} -dependent fluorescence after 30 minutes of agonist exposure and also effects of antagonists. Blockers of GluR6-containing receptors (CNQX and NS-102) were far more effective at reducing Ca^{2+} increase induced by kainate or SYM2081 than AMPA (spermine, GYKI52466) or GluR5 (UPB302) antagonists.

1-naphthyl acetyl spermine ($79 \pm 37\%$; $n = 49$; $p = 4 \times 10^{-10}$) or GYKI52466 ($83 \pm 18\%$; $n = 20$; $p = 7 \times 10^{-10}$), suggesting a partial activation of AMPA receptors by the latter agent at the concentrations used. UPB-302 was also less effective at blocking the SYM 2081 response ($94 \pm 40\%$; $n = 17$).

Ca^{2+} Stores Contribute to GluR6-Dependent Axonal Ca^{2+} Increase

To further characterize the sources of axonal Ca^{2+} increase, we applied agonists in the absence of bath Ca^{2+} ($+0.5\text{mM}$ EGTA), which reduced but did not completely prevent $F_{\text{Ca,ax}}$ increase (kainate + 0Ca^{2+} : $26 \pm 20\%$, $n = 33$, $p \approx 0$ vs Ca^{2+} -containing perfusate; SYM2081 + 0Ca^{2+} : $42 \pm 31\%$, $n = 24$, $p = 4 \times 10^{-10}$). This suggests that a component of the kainate receptor-induced axonal Ca^{2+} increase originated from

intracellular compartments. Previously, we reported that ischemic depolarization of spinal axons releases Ca^{2+} from ryanodine-dependent axonal Ca^{2+} stores.³⁰ We therefore examined whether kainate receptors might induce Ca^{2+} release from these stores. Ryanodine ($50\mu\text{M}$, in Ca^{2+} -replete perfusate) almost completely blocked the $F_{\text{Ca,ax}}$ increase (kainate + ryanodine: $2 \pm 22\%$, $n = 33$, $p = 0$ vs kainate alone; SYM2081 + ryanodine: $11 \pm 28\%$, $n = 27$, $p = 4 \times 10^{-10}$), indicating that most of the axonal Ca^{2+} accumulation observed in response to kainate receptor activation originated from axonal ryanodine-sensitive Ca^{2+} stores (Fig 2A). More surprisingly, blockade of L-type Ca^{2+} channels by nimodipine or nifedipine ($10\mu\text{M}$) also strongly inhibited axoplasmic Ca^{2+} increase (kainate + nimodipine: $6 \pm 19\%$, $n = 26$, $p \approx 0$ vs kainate alone; SYM2081 + nimodipine: $17 \pm$

Fig 2. Kainate receptors promote Ca^{2+} release from internal stores. (A) Ca^{2+} -free perfusate reduced but did not eliminate agonist-induced Ca^{2+} increase. Blocking of ryanodine receptors (ryanodine) strongly reduced Ca^{2+} response even in the presence of 2mM bath Ca^{2+} , suggesting that most of the agonist-induced Ca^{2+} increase was due to release from ryanodine-sensitive Ca^{2+} stores, rather than from influx across the axolemma. (B) L-type Ca^{2+} channel antagonists (nimodipine, nifedipine) selectively reduced responses induced by kainate or SYM2081. (C) Kainate receptor-dependent axonal Ca^{2+} increase depended on permeable ions such as Na^+ or Li^+ but was blocked by impermeable N-methyl-D-glucamine (NMDG). Error bars represent standard deviation.

22%, $n = 43$, $p = 4 \times 10^{-10}$) (see Fig 2B). L-type Ca^{2+} channels may, in turn, be modulated by a local membrane depolarization or possibly even by a metabotropic action of kainate receptors.³¹ Replacing NaCl with impermeant N-methyl-D-glucamine chloride (NMDG-Cl) to reduce putative agonist-induced axonal depolarization virtually abolished kainate- (kainate + NMDG: $-5 \pm 13\%$, $n = 37$, $p \approx 0$ vs Na^+ -containing perfusate) and SYM2081-induced Ca^{2+} increase (SYM2081 + NMDG: $15 \pm 17\%$, $n = 32$, $p = 1.7 \times 10^{-10}$). Substitution of NaCl with LiCl, which readily permeates kainate receptors,³² allowed a robust axonal Ca^{2+} increase after application of kainate ($91 \pm 50\%$; $n = 52$) or SYM2081 ($95 \pm 24\%$; $n = 34$) (see Fig 2C). Taken together, these data suggest that GluR6-containing kainate receptors mediate their actions through a combination of local membrane depolarization and a small influx of Ca^{2+} triggering a larger release from ryanodine-sensitive Ca^{2+} stores.

Intraaxonal Nitric Oxide Generation Promotes the Ca^{2+} Increase

Although the earlier results support the involvement of kainate receptors in the mobilization of Ca^{2+} , they do not prove that these receptors are necessarily axonal; indeed, the protective effects of AMPA/kainate antagonists in white matter injury was suggested to be due to protection of glial elements³³ with indirect sparing of axons (for review, see Matute and colleagues³⁴). The experiments shown in Figure 3A, relying on selective extracellular versus intraaxonal application of scavengers, strongly suggest that kainate receptors are expressed directly on axons and stimulate formation of nitric oxide (NO) within axons, which, in turn, promotes the above Ca^{2+} release cascade. Bath application of the NO scavenger myoglobin³⁵ failed to prevent axoplasmic Ca^{2+} increase (kainate + myoglobin: $80 \pm 66\%$, $n = 27$, $p = 0.2$ vs kainate alone; SYM2081 + myoglobin: $145 \pm 49\%$, $n = 34$, $p \approx 1$). Hydroxocobalamin, another NO scavenger³⁶ with a much smaller molecular weight (and, therefore, more readily able to permeate small interstitial spaces between axons, but nevertheless membrane impermeable), was equally ineffective (kainate + hydroxocobalamin: $90 \pm 71\%$, $n = 23$, $p = 0.94$ vs kainate alone). These experiments indicate that NO synthesized *outside* the axon did not play a role in kainate receptor-mediated Ca^{2+} release inside axons. To explore whether intraaxonally generated NO may be important, we selectively loaded myoglobin into axons. In contrast with bath application, intraaxonal scavenger potently blocked kainate- ($0 \pm 22\%$; $n = 22$) and SYM2081-induced ($16 \pm 33\%$; $n = 25$) Ca^{2+} responses ($p \approx 0$). Intraaxonal hydroxocobalamin was also highly effective, as was the nitric oxide synthase inhibitor L-NAME ($p \approx 0$). Moreover, the effect of intraaxonal NO was syner-

Fig 3. Kainate receptor-induced axonal Ca^{2+} response depends on intraaxonal nitric oxide (NO) generation. (A) Axonal Ca^{2+} increase was not reduced by extracellular application of NO scavengers (myoglobin, hydroxocobalamin). In contrast, intraaxonal loading of scavengers or of a nitric oxide synthase inhibitor (L-NAME) almost completely prevented the Ca^{2+} response. (B) Neither exposure to exogenous NO nor depolarization alone were sufficient to induce an axonal Ca^{2+} response. Combining an NO donor (250 μM PAPA NONOate) with 45 mM K^{+} produced a robust Ca^{2+} increase, which was dependent on activation of L-type Ca^{2+} channels and ryanodine receptors (* $p \leq 1.1 \times 10^{-5}$). Error bars represent standard deviation.

gistic with depolarization, even in the absence of receptor activation (see Fig 3B): Neither depolarization alone (45 mM K^{+} in the perfusate) nor exogenously applied NO (using the NO donor PAPA NONOate [250 μM]) induced an axonal Ca^{2+} increase. However, applying the NO donor during K^{+} -induced depolarization induced a substantial axonal Ca^{2+} increase,

which was greatly reduced by either nimodipine or ryanodine.

Axonal Signaling “Nanocomplexes” Containing GluR6/7, Neuronal Nitric Oxide Synthase, and $\text{Ca}_v1.2$

The previous observations suggest a close relation between axonally expressed GluR6 kainate receptors and nitric oxide synthase. Immunohistochemistry was performed to further localize these receptors and their associated signaling proteins (Fig 4). Punctate staining for GluR6/7 (using two different primary antibodies from different species) and nNOS was observed at the periphery of neurofilament-labeled axon cylinders. These clusters were often, but not invariably, colocalized. Although we did not attempt to examine the frequency of these complexes along the length of an axon, the representative micrograph in Figures 4A to C suggests that at least several clusters are present per internode. Immunoelectron microscopy localized GluR6/7 to the axolemma and to clusters beneath the axolemma. Consistent with earlier pharmacological evidence pointing to a functional interaction between kainate receptors and L-type Ca^{2+} channels, colocalized GluR6/7 and $\text{Ca}_v1.2$ clusters were also observed at the surfaces of axons (see Figs 4E–G). Immunoprecipitation of dorsal column lysate with the GluR6/7 antibody yielded a single nNOS-positive band indicating a physical association between this kainate receptor and the enzyme (see Fig 4I). We further hypothesized that a PDZ-binding motif on the C terminus of GluR6 may mediate an interaction between this receptor and an adaptor protein,³⁷ which, in turn, may scaffold the receptor in proximity to axonal nNOS to support a functional relation. We constructed a peptide comprising the nine C-terminal residues of GluR6 (RLPGKETMA, see Materials and Methods), to interfere with such a putative interaction. When this peptide was loaded into axons, both kainate and SYM2081 Ca^{2+} responses were almost completely blocked (kainate + peptide: $12 \pm 28\%$, $n = 77$, $p = 1.2 \times 10^{-5}$ vs kainate alone; SYM2081 + peptide: $13 \pm 27\%$, $n = 78$, $p = 1.1 \times 10^{-5}$). A sham peptide had little effect on the Ca^{2+} increase induced by kainate ($91 \pm 28\%$, $n = 45$) or SYM2081 ($96 \pm 30\%$; $n = 42$); the responses with the active compared with the sham peptides were highly significantly different ($p < 10^{-9}$ for both agonists) (Fig 5A). Further proof of an intraaxonal localization of a GluR6-PDZ domain, which could scaffold this receptor within a signaling nanocomplex containing nNOS, was obtained by loading the synthetic interfering peptide, itself labeled with multiple fluorescent moieties, into axons. As with the fixed immunohistochemical sections, we observed occasional punctate clusters of fluorescent peptide at the periphery of fluorescein-dextran-loaded axons (see Figs

Fig 4. Multimolecular “nanocomplexes” containing several signaling proteins are present in the internodal axolemma. (A–C) Triple-immunolabeled dorsal column axons showing occasional punctate regions of colocalized glutamate receptors 6 and 7 (GluR6/7) and neuronal nitric oxide synthase (nNOS) clusters (arrowheads) at the surface of neurofilament-stained axon cylinders. (inset) Transverse view of a surface cluster in another fiber. (D) Immunogold labeling using GluR6/7 primary antibody showing signal at the axolemma in a myelinated internode (arrow). my = myelin; ax = axon. Consistent with pharmacological manipulations and Ca^{2+} imaging (see Fig. 2), GluR6/7-containing clusters also colocalized with $\text{Ca}_v1.2$ L-type Ca^{2+} channels (E–G). (H) Representative control section with primary antibodies omitted showed little nonspecific labeling. Scale bars 2 μm . (I) GluR6/7 antibody immunoprecipitated nNOS as shown by the single nNOS-positive band at the expected molecular weight (IP GluR6/7). nNOS was also detected by straight immunoblotting in dorsal column lysate (raw DC lysate). As expected, control experiments with beads + lysate (without GluR6/7) or beads + GluR6/7 (without lysate) showed no nNOS signal (molecular weight markers in kilodaltons).

5B–D), consistent with the notion that these fibers contain discrete clusters of PDZ domains able to bind and likely cluster kainate receptors.

GluR6 Activation Causes Functional Dorsal Column Injury

Having identified such an arrangement of internodal signaling protein clusters capable of significantly increasing axonal Ca^{2+} levels, we then explored whether such persistent increases of Ca^{2+} had any functional implications in otherwise uninjured dorsal columns. Propagated compound action potentials were recorded electrophysiologically, and functional integrity of this white matter tract was determined by calculating the area under the digitized responses.³⁸ Exposure of dorsal columns to kainate (200 μM) or SYM2081 (100 μM) for 60 minutes followed by a 3-hour wash caused an irreversible reduction of mean compound action potential (CAP) area to approximately 60% of control (data not shown). Addition of the L-type Ca^{2+} channel blocker nimodipine (10 μM) significantly protected against kainate- (CAP area recovery: kainate + nimodipine, $93 \pm 17\%$, $n = 8$, vs kainate alone, $68 \pm$

10%, $n = 8$; $p = 0.003$, Wilcoxon rank test) and SYM2081-induced injury (SYM2081 + nimodipine, 83 ± 23 , $n = 9$, vs SYM2081 alone, 51 ± 15 , $n = 7$; $p = 0.0022$).

Discussion

A number of in vitro and in vivo studies have pointed to an important role for non-NMDA glutamate receptors in white matter injury,^{6,8,9,16} with glial cells representing an important target given their known expression of AMPA and kainate receptors,²⁰ and their sensitivity to this excitotoxin.^{24,39} This sensitivity to AMPA/kainate receptor activation also applies to immature oligodendrocyte precursors.⁴⁰ Glutamate is released from injured myelinated axons via reverse Na^+ -dependent glutamate transport⁷ and via vesicular release from unmyelinated fibers during physiological activation.^{41,42} In contrast, little is known about functional glutamate receptors on central axons, though experiments indirectly suggest that such receptors may be present.^{8,43}

Here we show that functional kainate receptors are present on myelinated central axons, raising the dis-

Fig 5. Infusing a peptide into axons that interfere with the binding of GluR6 to PDZ domains greatly reduced the Ca^{2+} response, whereas a sham peptide with the same sequence but synthesized using unnatural D-amino acids was far less effective (A). (B–D) Live spinal axons were coloaded with dextran fluorescein and Texas Red-conjugated peptide that recognizes a PDZ binding domain. Arrowhead shows a cluster of fluorescent peptide labeling an intraaxonal PDZ domain-containing protein complex. Scale bar = 2 μ m.

tinct possibility that loss of axonal function after glutamate exposure may also be caused by direct activation of axonal receptors leading to (possibly focal) axoplasmic Ca^{2+} deregulation. Curiously, immature premyelinated fibers are reported to suffer ischemic injury independently of glutamate receptors.³³ Contrasted with our findings in mature myelinated axons, this may indicate that myelination induces expression and clustering of axonal glutamate receptors, as it does other nodal and perinodal proteins.⁴⁴ Immunohistochemistry of dorsal column axons showed colocalized GluR6/7 and nNOS clusters sparsely distributed along axon cylinders as has been reported previously for Ca_v and RyR clusters.³⁰ Our results are consistent with the following proposed feed-forward mechanism (Fig 6): Activation of GluR6-containing kainate receptors induces a local depolarization of the internodal axolemma, together with a small amount of Ca^{2+} influx from a restricted periaxonal space. The local axonal Ca^{2+} microdomain promotes NO synthesis by nNOS, and the local depolarization activates L-type Ca^{2+} channels, thereby opening ryanodine receptors on subaxolemmal endoplasmic reticulum, culminating in a much larger Ca^{2+} transient than would be possible solely by influx of this ion. This is consistent with previous observations of kainate receptor-mediated depolarization of central axons.⁴³

Our electrophysiological recordings, which showed that functional injury induced by kainate receptor stimulation was significantly reduced by blocking L-type Ca^{2+} channels, emphasize two important points. First, given that activation of these receptors in otherwise uninjured dorsal columns results in significant functional impairment indicates that the observed Ca^{2+} increase induced by this treatment is pathophysiologically significant and raises the distinct possibility that exposure of axons to glutamate in inflammatory or ischemic lesions, for instance, may be directly damaging to axons. Second, the significant reduction in GluR6-mediated electrophysiological injury conferred by an L-type Ca^{2+} channel blocker further strengthens the functional connection between these receptors and Ca^{2+} channels, as suggested by the Ca^{2+} imaging experiments (see Fig 2) and summarized in the proposed model (see Fig 6).

The effect of NO is curious, though this modulator may function to increase the “gain” of the Ca_v -RyR coupling mechanism, possibly by upregulation of RyR activity.⁴⁵ This may be necessary to ensure the fidelity of this signaling cascade, because unlike neurons and muscle cells that are not ensheathed, voltage-gated proteins such as Ca_v s, which are localized to the internodal axolemma of myelinated fibers, likely experience smaller electric-field fluctuations because of the overlying myelin. Given the known promiscuous actions of NO (and its highly reactive derivative peroxynitrite), it is possible that other ion transporters, which are important for axonal impulse propagation (eg, voltage-gated Na and K channels, Na-K-ATPase⁴⁶), may be modulated as well in response to kainate receptor/nNOS activation. Thus, central myelinated axons contain functional complexes of several signaling proteins that are arranged in close proximity (eg, GluR6/7, nNOS, and $\text{Ca}_v1.2$; see Fig 4; L-type Ca^{2+} channels and ryanodine receptors³⁰), allowing local NO production and depolarization to modulate their function. The purpose of such clusters in mature myelinated fibers is currently unknown; in developing axons, however, growth cone dynamics have been shown to be dependent on glutamate receptor activation and release of Ca^{2+} from intraaxonal Ca^{2+} stores,⁴⁷ indicating that ionotropic glutamate receptors and Ca^{2+} signaling from axonal stores are functionally related from an early developmental age. Their precise physiological roles in adulthood will require further study. Scaffolding of axonal receptors and effectors such as nNOS in close proximity is reminiscent of the organization of signaling molecules at the postsynaptic density in neurons,^{48,49} and it hints at highly specialized and complex machinery assembled along the internodal axolemma, where little active signaling was thought to take place.

Both glutamate- and NO-dependent toxicity are in-

Fig 6. Proposed arrangement of signaling molecules in internodal axonal nanocomplexes. GluR4 AMPA receptors permeate small amounts of Ca^{2+} , which, in turn, release Ca^{2+} from the axoplasmic reticulum (AR) via “cardiac-type” Ca^{2+} -induced Ca^{2+} release.⁵¹ Axonal Ca^{2+} increases from activation of GluR5 kainate receptors occur mainly via a G-protein-coupled, phospholipase C (PLC)-dependent synthesis of IP_3 , which, in turn, activates IP_3 receptors on the AR; this latter mechanism is partially dependent on NO, which is synthesized by nNOS, itself activated by small amounts of Ca^{2+} entry via the GluR5 receptor; the locally produced NO may then further upregulate IP_3 receptor activity.⁵¹ Activation of GluR6 kainate receptors induces a local depolarization and a small amount of Ca^{2+} entry. The depolarization activates L-type Ca^{2+} channels (Ca_v), whereas the kainate receptor-mediated Ca^{2+} influx stimulates nNOS, which is scaffolded in the vicinity of the receptor. Similarly to GluR5 receptors, locally generated NO may upregulate the activity of ryanodine receptors, which are activated by the depolarization-induced conformational change of the Ca^{2+} channel, leading to release of Ca^{2+} from the AR. Together, these mechanisms, possibly activated by axonally released glutamate, serve to amplify the axonal Ca^{2+} signal, which would normally be weak because of the limited quantity of ion available in the narrow periaxonal space.

involved in white matter injury, and particularly in axonal damage, in crippling disorders such as multiple sclerosis.³⁴ The signaling clusters described in this report likely promote and amplify local Ca^{2+} transients, and may have profound implications for axonal pathophysiology. The local release of potentially high concentrations of Ca^{2+} through activation of such axonal “nanocomplexes” may play an important role in the genesis of focal swellings and irreversible axonal transections⁵⁰ that render the entire fiber nonfunctional. The surprisingly complex interaction of glutamate, NO, voltage-gated Ca^{2+} channels, and internal Ca^{2+} stores in axons may paradoxically present unforeseen opportunities for the development of novel therapeutic strategies.

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