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 Ca2+ deregulation.

Methods: Dorsal column axons were loaded with a Ca2+ 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 [Ca2+]. This Ca2+ 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 Ca2+ channels: ryanodine, nimodipine, or nifedipine blocked the agonist-induced Ca2+ increase. Also, GluR6 stimulation induced intraxonal production of nitric oxide (NO), which greatly enhanced the Ca2+ response: quenching of NO with intraaxonal (but not extracellular) scavengers, or inhibition of neuronal NO synthase with intraaxonal N-nitro-L-arginine methyl ester, blocked the Ca2+ 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 Ca2+ increase. Immunohistochemistry showed GluR6/7 clusters on the axolemma colocalized with neuronal NO synthase and Cav1.2.

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 Ca2+ 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.

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 Ca2+ channels, together with reverse Na+-Ca2+ exchange, play important roles2–4 (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 vitro6–10 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 vitro15–17 or in vivo18–20 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,
NMDA receptors have been detected on mature oligodendrocytes,21 their processes,22 and even the myelin sheath.23 These receptors are permeable to Ca\textsuperscript{2+} ions; therefore, it is reasonable to conclude that receptor-mediated Ca\textsuperscript{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.6,13,26 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 NMDA, 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\textsuperscript{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\textsuperscript{2+} channels and causing Ca\textsuperscript{2+} release from intraxonal Ca\textsuperscript{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\textsuperscript{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\textsuperscript{2+} solution (containing in mM: NaCl 126, KCl 3, MgSO\textsubscript{4} 2, NaHCO\textsubscript{3} 26, NaH2PO\textsubscript{4} 1.25, MgCl\textsubscript{2} 2, dextrose 10 and EGTA 0.5, oxygenated with 95% O\textsubscript{2}/5% CO\textsubscript{2}), loaded for 2 hours with Ca\textsuperscript{2+}-insensitive reference dye (red dextran-conjugated Alexa 594, 250\textmu M) to allow identification of axon profiles (Fig 1A), together with the dextran-conjugated Ca\textsuperscript{2+} indicator Oregon Green BAPTA-1 (250\textmu M), and imaged on a Nikon C1 (Toronto, Ontario) confocal microscope at 37°C. All reported axonal [Ca\textsuperscript{2+}] changes (F\textsubscript{Ca,ax}) are ratios of green to red fluorescence after 30 minutes of drug application.

**Immunohistochemistry and Immunoelectron Microscopy**

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

**Peptide Synthesis and Purification**

Two peptides (NH\textsubscript{2}-Cys-Ahx-Arg-Leu-Pro-Gly-Lys-Glu-Thr-Met-Ala-CONH\textsubscript{2}, (I), [molecular weight = 1,218] and NH\textsubscript{2}-Cys-Ahx-Cys-Ahx-Cys-Ahx-Ahx-Ahx-Arg-Leu-Pro-Gly-Lys-Glu-Thr-Met-Ala-CONH\textsubscript{2}, (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 (\epsilon-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 10\textmu M in the loading pipette yielding approximately 1 to 10\textmu M in the axons.

**Results**

**Activation of GluR6-Containing Receptors Increases Axonal Ca\textsuperscript{2+}**

We measured [Ca\textsuperscript{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\textmu M), at concentrations that significantly reduced compound action potentials (see later), caused a progressive increase of intraxonal [Ca\textsuperscript{2+}]. Axoplasmic Ca\textsuperscript{2+}-dependent fluorescence (F\textsubscript{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\textmu M) (12 \pm 15%; n = 35; p \approx 0). The AMPA receptor antagonists 1-naphthyl acetyl spermine (25\textmu M) or 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5H-pyrido[2,3-d]pyrimidin-2-one (NS-102 10\textmu M) did not significantly blunt kainate-induced F\textsubscript{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\textmu M), 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\textmu M), 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\textsuperscript{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\textmu M), another kainate receptor agonist,29 induced an increase of F\textsubscript{Ca,ax} (135 \pm 67%; n = 79) with a similar pharmacological profile to kainate: The Ca\textsuperscript{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
1-naphthyl acetyl spermine (79\% ± 37\%; n = 49; p = 4 × 10^{-10}) or GYKI52466 (83\% ± 18\%; n = 20; p = 7 × 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 SYM2081 response (94\% ± 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+} (+0.5mM EGTA), which reduced but did not completely prevent F_{\text{Ca,ax}} increase (kainate + 0Ca^{2+}: 26 ± 20\%, n = 33, p ≈ 0 vs Ca^{2+}-containing perfusate; SYM2081 + 0Ca^{2+}: 42 ± 31\%, n = 24, p = 4 × 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.\footnote{30}

We therefore examined whether kainate receptors might induce Ca^{2+} release from these stores. Ryanodine (50\mu M, in Ca^{2+}-replete perfusate) almost completely blocked the F_{\text{Ca,ax}} increase (kainate + ryanodine: 2 ± 22\%, n = 33, p = 0 vs kainate alone; SYM2081 + ryanodine: 11 ± 28\%, n = 27, p = 4 × 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 M) also strongly inhibited axoplasmic Ca^{2+} increase (kainate + nimodipine: 6 ± 19\%, n = 26, p ≈ 0 vs kainate alone; SYM2081 + nimodipine: 17 ±
22%, n = 43, p = 4 × 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. Replacing NaCl with impermeant N-methyl-D-glucamine chloride (NMDG-Cl) to reduce putative agonist-induced axonal depolarization virtually abolished kainate- (kainate + NMDG: −5 ± 13%, n = 37, p = 0 vs Na^+ -containing perfusate) and SYM2081-induced Ca^{2+} increase (SYM2081 + NMDG: 15 ± 17%, n = 32, p = 1.7 × 10^{-10}). Substitution of NaCl with LiCl, which readily permeates kainate receptors, allowed a robust axonal Ca^{2+} increase after application of kainate (91 ± 50%; n = 52) or SYM2081 (95 ± 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 elements with indirect sparing of axons (for review, see Matute and colleagues). 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 myoglobin failed to prevent axoplasmic Ca^{2+} increase (kainate + myoglobin: 80 ± 66%, n = 27, p = 0.2 vs kainate alone; SYM2081 + myoglobin: 145 ± 49%, n = 34, p ≈ 1). Hydroxocobalamin, another NO scavenger 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 ± 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 ± 22%; n = 22) and SYM2081-induced (16 ± 33%; n = 25) Ca^{2+} responses (p ≈ 0). Intraaxonal hydroxocobalamin was also highly effective, as was the nitric oxide synthase inhibitor L-NAME (p ≈ 0). Moreover, the effect of intraaxonal NO was syner-
gistic with depolarization, even in the absence of receptor activation (see Fig 3B): Neither depolarization alone (45mM K\(^+\) in the perfusate) nor exogenously applied NO (using the NO donor PAPA NONOate [250\(\mu\)M]) 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 Ca\(_{\text{a,1.2}}\)

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 Ca\(_{\text{a,1.2}}\) 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^{-3} for both agonists) (Fig 5A). Further proof of an intraxon- onal 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 A and B).
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. Exposure of dorsal columns to kainate (200\(\mu\)M) or SYM2081 (100\(\mu\)M) for 60 minutes followed by a 3-hour wash caused an irreversible reduction of mean compound action potential area to approximately 60% of control (data not shown). Addition of the L-type Ca\(^{2+}\) channel blocker nimodipine (10\(\mu\)M) significantly protected against kainate- (CAP area recovery: kainate + nimodipine, 93 ± 17%, n = 8, vs kainate alone, 68 ± 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, with glial cells representing an important target given their known expression of AMPA and kainate receptors, and their sensitivity to this excitotoxin. Glutamate is released from injured myelinated axons via reverse Na\(^{+}\)-dependent glutamate transport and via vesicular release from unmyelinated fibers during physiological activation. In contrast, little is known about functional glutamate receptors on central axons, though experiments indirectly suggest that such receptors may be present.

Here we show that functional kainate receptors are present on myelinated central axons, raising the dis-
larization of central axons.43 Previous observations of kainate receptor–mediated depolarization of axonal receptors leading to (possibly focal) 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 Ca2+ 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 Ca2+ influx from a restricted periaxonal space. The local axonal Ca2+ microdomain promotes NO synthesis by nNOS, and the local depolarization activates L-type Ca2+ channels, thereby opening ryanodine receptors on subaxolemmal endoplasmic reticulum, culminating in a much larger Ca2+ 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 Ca2+ 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 Ca2+ 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 Ca2+ channel blocker further strengthens the functional connection between these receptors and Ca2+ channels, as suggested by the Ca2+ 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 Ca2+-RyR coupling mechanism, possibly by upregulation of RyR activity.53 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 Ca2+, 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 Ca2+; see Fig 4; L-type Ca2+ 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 Ca2+ from intraxonal Ca2+ stores,47 indicating that ionotropic glutamate receptors and Ca2+ 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-

![Image](58x512 to 286x731)

Fig 5. Infusing a peptide into axons that interfere with the binding of GluR6 to PDZ domains greatly reduced the Ca2+ 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 μm.
involved in white matter injury, and particularly in axonal damage, in crippling disorders such as multiple sclerosis. The signaling clusters described in this report likely promote and amplify local Ca\(^{2+}\)/H\(_{11001}\) transients, and may have profound implications for axonal pathophysiology. The local release of potentially high concentrations of Ca\(^{2+}\)/H\(_{11001}\) through activation of such axonal “nanocomplexes” may play an important role in the genesis of focal swellings and irreversible axonal transections that render the entire fiber nonfunctional.

The surprisingly complex interaction of glutamate, NO, voltage-gated Ca\(^{2+}\)/H\(_{11001}\) channels, and internal Ca\(^{2+}\)/H\(_{11001}\) stores in axons may paradoxically present unforeseen opportunities for the development of novel therapeutic strategies.

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