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Acrolein-mediated conduction loss is partially restored by K^+ channel blockers

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Yan R, Page JC, Shi R. Acrolein-mediated conduction loss is partially restored by K^+ channel blockers. *J Neurophysiol* 115: 701–710, 2016. First published November 18, 2015; doi:10.1152/jn.00467.2015.—Acrolein-mediated myelin damage is thought to be a critical mechanism leading to conduction failure following neurotrauma and neurodegenerative diseases. The exposure and activation of juxtaparanodal voltage-gated K^+ channels due to myelin damage leads to conduction block, and K^+ channel blockers have long been studied as a means for restoring axonal conduction in spinal cord injury (SCI) and multiple sclerosis (MS). In this study, we have found that 100 μM K^+ channel blockers 4-aminopyridine-3-methanol (4-AP-3-MeOH), and to a lesser degree 4-aminopyridine (4-AP), can significantly restore compound action potential (CAP) conduction in spinal cord tissue following acrolein-mediated myelin damage using a well-established ex vivo SCI model. In addition, 4-AP-3-MeOH can effectively restore CAP conduction in acrolein-damaged axons with a range of concentrations from 0.1 to 100 μM. We have also shown that while both compounds at 100 μM showed no preference of small- and large-caliber axons when restoring CAP conduction, 4-AP-3-MeOH, unlike 4-AP, is able to augment CAP amplitude while causing little change in axonal responsiveness measured in refractory periods and response to repetitive stimuli. In a prior study, we show that 4-AP-3-MeOH was able to functionally rescue mechanically injured axons. In this investigation, we conclude that 4-AP-3-MeOH is an effective K^+ channel blocker in restoring axonal conduction following both primary (physical) and secondary (chemical) insults. These findings also suggest that 4-AP-3-MeOH is a viable alternative of 4-AP for treating myelin damage and improving function following central nervous system trauma and neurodegenerative diseases.

demyelination; spinal cord injury; hydralazine; 4-aminopyridine; potassium ion

MYELIN PERMITS RAPID AXONAL conduction in the nervous system that is essential for proper motor and sensory function. As an effective insulator, the myelin sheath wraps around axons and facilitates fast propagation of action potentials (AP) called saltatory conduction (Poliak and Peles 2003; Shi and Sun 2011). The unique demarcation of channel expression, Na^+ channels in the nodes of Ranvier where myelin is absent and K^+ channels in the juxtaparanodal regions that are myelinated, ensures that APs are triggered at and propagate between the nodes (Leung et al. 2011a; Ouyang et al. 2010; Poliak and Peles 2003; Shi et al. 2015; Shi and Sun 2011; Sun et al. 2010). However, while such a delicate structure is responsible for efficient transmission of electrical signals that are critical for physiological function, it renders the myelinated axon vulnerable to physical and chemical damages that could compromise nerve conduction, a common characteristic of many neuropathologies (Blight 1985; Compston and Coles 2008; Rudick and Trapp 2009; Shi et al. 2015; Shi and Sun 2011; Totoiu and Keirstead 2005). In particular, it has been shown that mechanical forces, including compression and stretch, and chemical factors, such as oxidative stress, inflammation, or glutamate excitotoxicity, could cause various types of myelin damage and consequential conduction failure (Babbs and Shi 2013; Fu et al. 2009; Jensen and Shi 2003; Lima et al. 2008; Micu et al. 2006; Ouyang et al. 2010; Shi and Blight 1997; Shi and Sun 2011; Shi et al. 2011b; Smith et al. 1999).

While the etiology of myelin damage is incompletely understood, the mechanism by which demyelination leads to axonal conduction loss is relatively clear. A current leading mechanism is that the unmasking of juxtaparanodal voltage-gated K^+ channels subsequent to pathological myelin retraction can prevent adequate depolarization of the nodal region due to the efflux of K^+, the intra-axonal positive charge carrier, through these channels (Ouyang et al. 2010; Shi and Sun 2011; Sun et al. 2012; Sun et al. 2010). Resulting insufficient depolarization at the node would prevent the sufficient activation of voltage-gated Na^+ channels and lead to the failure of generation and therefore conduction of APs. Such understanding has led to the development of effective pharmaceutical interventions to restore the axonal electrical impulse conduction by blocking exposed K^+ channels in both animal experimentation and clinical application (Blight 1989; Blight and Gruner 1987; Blight et al. 1991; Blight and Tuszynski 2006; Jensen and Shi 2003; Leung et al. 2011a; Shi and Blight 1997; Shi et al. 1997; Sun et al. 2010; Young 1993). 4-Aminopyridine (4-AP), a known K^+ channel blocker, has been shown to effectively restore conduction in demyelinated axons in animal studies and has subsequently gained Food and Drug Administration (FDA) approval to improve motor function in multiple sclerosis (MS), a neurodegenerative disease marked by myelin damage and axonal conduction loss (Blight 2011; Blight et al. 2014; Jensen et al. 2014). However, the ability of 4-AP to restore function, although significant, has been limited. This is likely due to its narrow achievable therapeutic range near 1 μM. The potential adverse side effects could surface when blood levels exceed 1 μM (Bever 1994; Halter et al. 2000). In light of the success of this strategy and the limitations of 4-AP, novel K^+ channel blockers have been developed as possible alternatives for functional restoration (McBride et al. 2006, 2007; Smith et al. 2005). Among them, one compound, 4-aminopyridine-3-methanol (4-AP-3-MeOH), has been shown to block the same K^+

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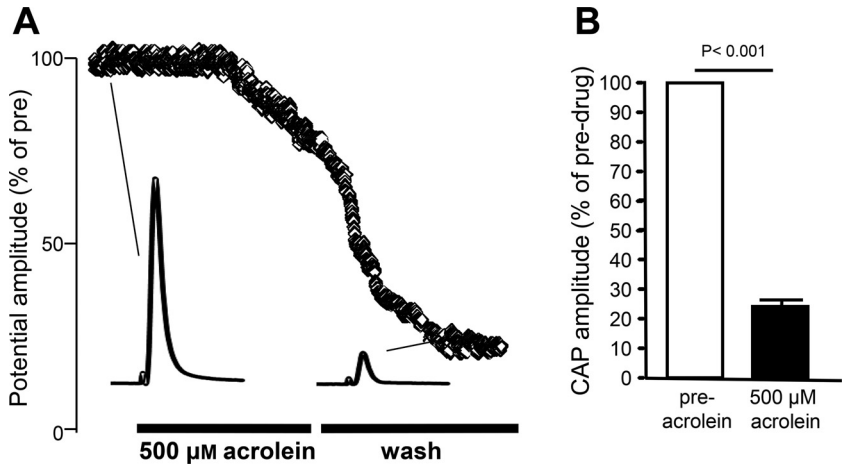

Fig. 1. Reduction of compound action potential (CAP) amplitude with acrolein in ex vivo spinal cord ventral white matter strips. *A*: the trend line depicts a typical CAP response to acrolein exposure (500 μM, 60 min) followed by washout with normal Krebs' solution (60 min). Representative CAP waveforms are shown for pre- and postacrolein exposure. *B*: the bar graph shows the CAP amplitude normalized to preacrolein in two conditions: before acrolein exposure (preacrolein) and after the exposure to acrolein (500 μM acrolein). Note that a significant reduction in amplitude results from acrolein exposure ($P < 0.001$, $n = 32$). Error bars represent SE.

channels that are targeted by 4-AP and restore axonal conduction on mechanically induced demyelination (Leung et al. 2011a; Sun et al. 2010). However, the benefit of 4-AP-3-MeOH in chemically induced myelin damage has yet to be investigated.

While it is well-known that oxidative stress is a major chemical causal factor for myelin damage, it has only recently emerged that acrolein, a highly reactive aldehyde and key factor and perpetuator of oxidative stress, is capable of causing significant myelin damage (Hamann et al. 2008a, 2008b; Park et al. 2014b; Shi et al. 2011b, 2015; Smith et al. 1999). Acrolein has been specifically shown to inflict paranodal myelin retraction in ex vivo spinal cord studies (Shi et al. 2011b), which is a key pathological factor also identified in the well-established animal model of MS autoimmune encephalomyelitis (EAE) (Leung et al. 2011b). Specifically, acrolein is elevated in EAE, and its mitigation was associated with marked alleviation of myelin damage and associated symptoms (Leung et al. 2011b; Shi et al. 2015; Tully et al. 2014). Therefore, acrolein appears to be a major contributor of myelin damage in the animal model of MS. In light of these recent developments,

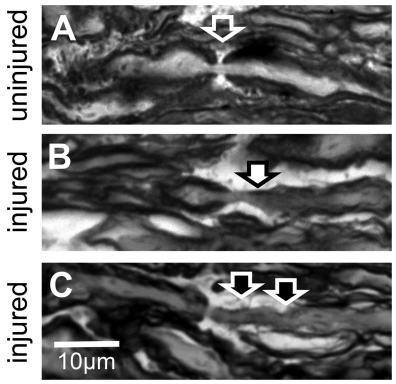

Fig. 2. Paranodal demyelination in spinal cord white matter following acrolein exposure. Photographs were taken from toluidine blue-stained, 0.5-μm plastic sections. *A*: longitudinal section from a normal uninjured spinal cord ventral white matter exhibit tight adhesion of paranodal myelin to the axolemma directly adjacent to the node of Ranvier and symmetric myelin structure on either side (arrow). *B*: image of a similar section from an isolated tract that was exposed to acrolein displays myelin detachment from axolemma in the paranodal region and elongation of the node of Ranvier (arrow). Note the striking asymmetry in the myelin thickness on either side of a node of Ranvier. *C*: a similar segment from another spinal cord shows more prominent myelin damage as represented by the increased length of the node of Ranvier and near absence of myelin structure at one side of the axon (arrows).

we wanted to ascertain the effectiveness of 4-AP-3-MeOH for functional restoration following acrolein-induced demyelination and its comparison with the existing K^+ channel blocker 4-AP, an effort that has not been attempted before. Our data suggest that 4-AP-3-MeOH is effective in restoring axonal conduction in acrolein-damaged ex vivo spinal cord tissue. Such conduction restoration seems to be superior to 4-AP both in the magnitude of compound action potential (CAP) conduction enhancement and the minimal disturbance of refractoriness in response to dual and multiple stimuli. Therefore, 4-AP-3-MeOH appears to be a viable alternative to 4-AP for symptomatic alleviation of function loss in neurotrauma and neurodegenerative diseases particularly where acrolein and myelin damage have been implicated.

METHODS

Spinal Cord Isolation

All animals were handled and housed in accordance with the protocol submitted to and independently reviewed and approved by the Purdue Animal Care and Use Committee (PACUC). Male Sprague-Dawley rats ranging from 200 to 400 g were used. Anesthetized with a combination of ketamine (80 mg/kg) and xylazine (10 mg/kg) through intraperitoneal injection, rats were tested for diminished sensory function with a toe pinch before surgery. After exposure of the thoracic cavity, a transcardial perfusion was performed with cold, oxygenated Krebs' solution (in mM): 124 NaCl, 2 KCl, 1.2 KH_2PO_4, 1.3 $MgSO_4$, 1.2 $CaCl_2$, 10 dextrose, 5.6 sodium ascorbate, and 26 $NaHCO_3$. The vertebrae column was then excised, and a complete dorsal laminectomy was performed to allow for careful removal of the spinal cord. The spinal cord itself was dissected into lateral segments along the midline before being cut in half, and finally separated into dorsal and ventral segments. Only ventral segments were saved and stored in Krebs' solution, which was bubbled continuously with 95% O_2-5% CO_2 to maintain a pH of 7.2–7.4 and kept at room temperature. Spinal cord segments were allowed to recover for 1 h after surgery before being used for electrophysiology recordings.

Double-Sucrose Gap Recordings

Ventral spinal cord segments were placed in a double-sucrose gap recording apparatus to acquire functional measurements. The apparatus contains the following five wells: one central, two neighboring, and two outer that contain continuously perfused oxygenated Krebs solution, isotonic sucrose (320 mM), and isotonic potassium chloride (120 mM), respectively. Each compartment is sealed from the neighboring ones with thin plastic sheets and vacuum grease to prevent

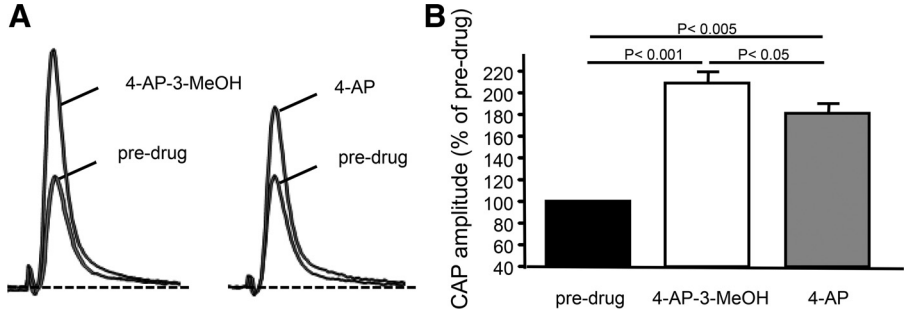

Fig. 3. Augmentation of CAP amplitude in response to 4-aminopyridine (4-AP) and 4-aminopyridine-3-methanol (4-AP-3-MeOH) following an acrolein-mediated conduction decrease in ex vivo spinal cord white mater. *A*: examples of CAP waveforms of acrolein-treated cord samples before (predrug) and 60 min post-4-AP (100 μM) or 4-AP-3-MeOH (100 μM) application. Note the more pronounced increase of CAP amplitude in 4-AP-3-MeOH-treated cord samples compared with that of 4-AP. *B*: graph displays quantitative analysis of the change of CAP amplitude in response to 4-AP or 4-AP-3-MeOH as a percentage of predrug. A significant increase from predrug is observed with application of 4-AP ($P < 0.005$, $n = 10$) or 4-AP-3-MeOH ($P < 0.001$, $n = 10$). Moreover, the increase of CAP amplitude from 4-AP-3-MeOH is greater than that from 4-AP ($P < 0.05$, $n = 10$). Error bars represent SE.

exchange of solutions. Axons were stimulated with 0.1-ms constant-current unipolar pulses. A bridge amplifier (Neurodata Instruments) was used to acquire recordings, and later analysis was conducted using custom Labview software (National Instruments) on a Dell personal computer (PC). Additional information can be located in previous publications (Shi and Blight 1996; Shi and Borgens 1999; Shi and Pryor 2002).

CAP amplitude. Spatiotemporal summations of many single-unit APs fired by individual axons comprise each CAP. Constant application of 0.1-ms unipolar pulses was delivered every 3 s with a supermaximal stimulus (110% of the maximal stimulus). CAP profiles were obtained by averaging multiple recordings at predetermined times and used for subsequent analysis. A real-time plot of the CAP amplitude was also recorded throughout each experiment and saved in a PC for further reference (Jensen and Shi 2003; Shi and Blight 1996; Shi and Borgens 1999; Shi and Pryor 2002).

Activation threshold. The threshold for firing APs in each axon is determined in part by its caliber (BeMent and Ranck 1969; Jensen and Shi 2003; West and Wolstencroft 1983). Gradual stimulation of axons with different thresholds can be accomplished by current-voltage tests where larger-diameter axons will theoretically conduct first. Stimulus intensities ranging from 1.85 to 6.5 volts were applied before and after drug administration. A total of five stimuli were applied at specific stimulus intensities, averaged, and used for subsequent analysis. Stimuli were delivered with a 3-s frequency throughout the recording.

Dual-stimuli refractory period response. Absolute and relative refractory periods of spinal cord white matter were determined using twin pulse stimuli at 110% of the maximal stimulus with a varying interstimulus range of 0.5 to 13 ms. The amplitude of the initial CAP remained constant for each paired stimuli. The time required for a second CAP to fire is considered the absolute refractory period while the time required for the CAP amplitude to reach 95% of the first is defined as the relative refractory period. Dual stimuli were applied both predrug and after administration of each compound.

Multiple-stimuli response. Application of train stimuli at low (500 Hz) and high (1,000 Hz) frequencies for 100 ms was delivered to spinal cord white matter tissue. An average of the final four CAPs is expressed as a percentage of the initial CAP for both predrug and following K^+ channel blocker application.

Acrolein-Mediated Injury

Electrophysiology. Spinal cord ventral white matter segments placed in a double-sucrose gap recording chamber were perfused with 500 μM acrolein in normal oxygenated Krebs' solution at 37°C. Acrolein was added to the solution shortly before perfusion, and the cord was subsequently perfused for 60 min.

Morphology. Submersion of spinal cord ventral white matter segments in 500 μM acrolein was conducted for 12 h. The solution was continuously oxygenated and replaced every 2 h to ensure removal of toxic metabolites.

Plastic Sections

Spinal cord ventral white matter sections were incubated in oxygenated solution with 500 μM acrolein for 12 h to allow for acrolein-mediated injury. Similarly, control uninjured sections were kept in oxygenated normal Krebs' solution for 12 h. Following incubation, the tissue was fixed with a 3% glutaraldehyde solution as the primary fixative in 0.1 M sodium cacodylate trihydrate ($C_2H_6AsNaO_2 \cdot 3H_2O$) working buffer. Longitudinal sections were cut while tissue was submersed in the primary fixative and then washed with the working buffer and distilled water. Sections were postfixed in 1% reduced osmium tetroxide in the secondary fixative, 1.5% potassium ferricyanide, and washed with distilled water. Following fixation, samples were dehydrated with ethanol (EtOH) or acetone, infiltrated with propylene oxide if dehydrated with EtOH, embedded with 100% resin, and polymerized at 60°C for 48 h. Resin blocks were cut into 1.5-μm-thick plastic sections using a Porter-Blum ultramicrotome and stained with 1% toluidine blue. Representative images were captured with an Olympus BX61 microscope (Olympus Scientific, Melville, NY).

Chemical Agents

Acrolein (Sigma, St. Louis, MO), 4-AP (Sigma), and 4-AP-3-MeOH (Alfa Aesar, Ward Hill, MA) were applied to spinal cord

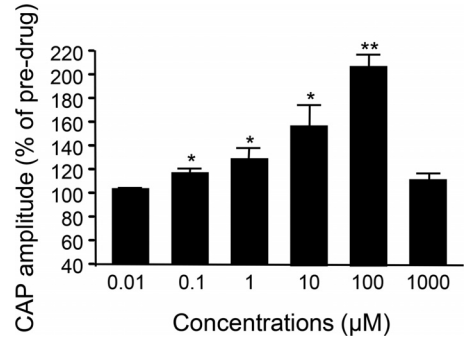

Fig. 4. The increase of CAP amplitude in response to different concentrations of 4-AP-3-MeOH in spinal cord tissue injured by acrolein exposure. The augmentation in CAP amplitude following 4-AP-3-MeOH application for 60 min was normalized to the pre-4-AP-3-MeOH level. CAP amplitude significantly increased in 0.1, 1, 10, and 100 μM of 4-AP-3-MeOH ($n = 5$–10; *$P < 0.05$ and **$P < 0.001$). 4-AP-3-MeOH at 0.01 μM and 1 mM did not significantly enhance CAP amplitude. All concentration groups were compared with control with a paired *t*-test. Error bars represent SE.

sections. Each compound was dissolved in normal Krebs' solution immediately preceding perfusion in the central compartment of the double-sucrose gap recording apparatus. Each solution was continuously oxygenated and maintained at 37°C. The following concentrations of each compound were used: 500 μM acrolein, 100 μM 4-AP, and 100 μM 4-AP-3-MeOH (Hamann et al. 2008a, 2008b; Shi et al. 1997; Shi and Sun 2011; Sun et al. 2010).

Statistical Analysis

ANOVA and Tukey-Kramer tests were used to compare the data related to the changes of the amplitude of CAP where multiple comparisons were made (Fig. 3*B*). A Student's *t*-test was used in all other analyses where a single comparison between two groups was made. Statistical significance was attained with values where $P < 0.05$ and averages were expressed as $\pm$ SE.

RESULTS

Acrolein Causes Conduction Loss in Normal Spinal Cord White Matter

Spinal cord ventral white matter segments were placed in a double-sucrose gap recording chamber for functional assessment during acrolein treatment. Following a 30- to 60-min stabilization period, 500 μM acrolein was applied to the bath for 60 min and then washed out for another 60 min with normal Krebs' solution. Real-time measurement of the CAP amplitude, measured as a percentage of preacrolein, demonstrates a progressive decline of CAP amplitude due to administration of acrolein (Fig. 1*A*). Example CAP waveforms provide visual representation of this reduction in amplitude. Measurement of CAP amplitude after 60 min acrolein treatments shows a significant decrease in amplitude from preacrolein: 100% to 23.3 $\pm$ 1.9% ($P < 0.001$, $n = 32$) (Fig. 1*B*).

Acrolein Causes Morphological Asymmetric Paranodal Demyelination

Subsequent to the functional electrophysiological assessments aforementioned, an anatomical investigation of the spinal cord tissue segments was conducted. Toluidine blue was applied to 1.5-μm plastic sections of normal uninjured tissue and those with acrolein-mediated injury for comparison. A longitudinal section of normal tissue reveals an identifiable node of Ranvier and myelin tightly wrapped symmetrically around paranodal and internodal axonal regions (Fig. 2*A*). In

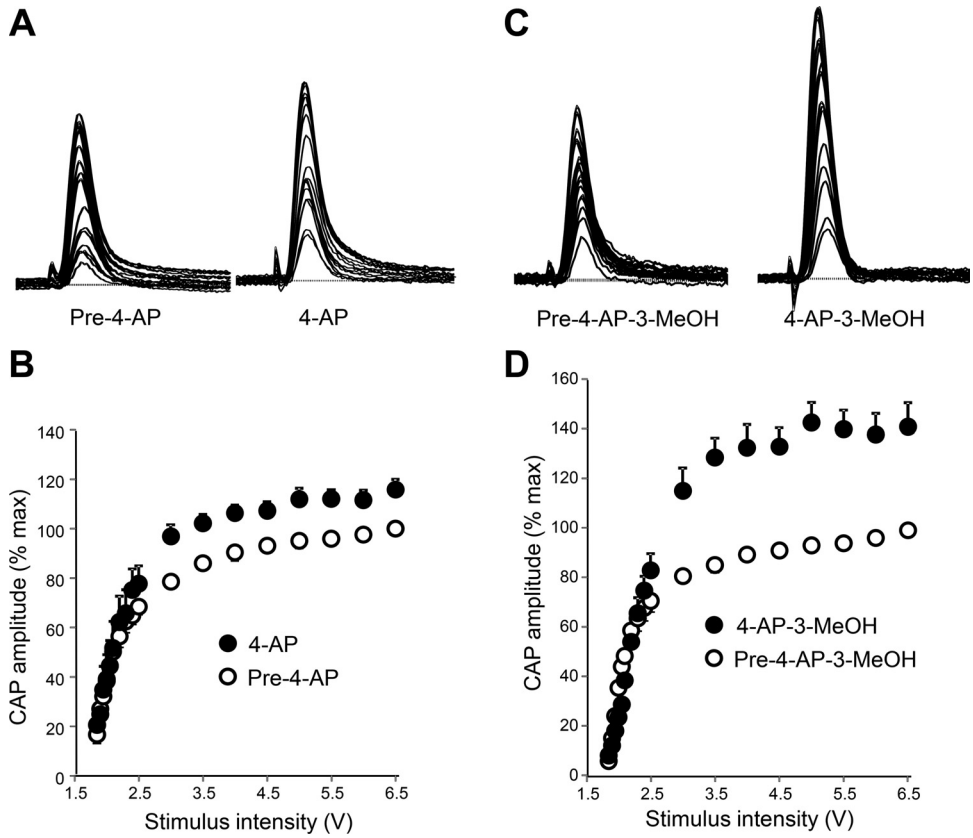

Fig. 5. Assessment of activation threshold changes as a result of 4-AP (*A* and *B*) or 4-AP-3-MeOH (*C* and *D*) application to acrolein-injured cord white matter samples. Activation threshold was determined based on CAP responses at different stimulus intensities at two conditions: predrug or drug (4-AP or 4-AP-3-MeOH). Superimposed CAP waveforms (*A*) and a graph plot form (*B*) are shown for pre-4-AP or 4-AP for the purpose of comparison. Ventral white matter spinal cord segments were stimulated over a range of intensities from 1.85 to 6.5 V. Each data point demonstrates the average of five segments. CAP amplitudes were normalized as a percentage of maximum CAP amplitude in the pre-4-AP condition. Note a significant increase in the CAP amplitude from pretreated (pre-4-AP) segments resulted from 4-AP application over the stimulation range of 2.5–6.5 V ($P < 0.05$, $n = 5$). Error bars represent SE. Superimposed CAP waveforms (*C*) and a graph plot form (*D*) are shown for pre-4-AP-3-MeOH and 4-AP-3-MeOH for the purpose of comparison. Similar to *A* and *B*, ventral white matter spinal cord segments were stimulated over a range of intensities from 1.85 to 6.5 V. Each data point demonstrates the average of five segments. CAP amplitudes were normalized as a percentage of maximum CAP amplitude in the pre-4-AP-3-MeOH condition. A significant increase in the CAP amplitude from pretreated (pre-4-AP-3-MeOH) segments resulted from 4-AP-3-MeOH application over the stimulation range of 2.5–6.5 V ($P < 0.05$, $n = 5$). Error bars represent SE.

the presence of acrolein-mediated injury, structural changes occurred, including decompaction, retraction, and disruption of myelin from the paranodal axonal membrane region resulting in elongated nodes and exposed axonal regions (Fig. 2*B*). Compared with Fig. 2*B*, the severity of the damage is even more pronounced in Fig. 2*C* where a similar segment of spinal cord tissue shows extensive asymmetric demyelination from the axolemma.

K^+ Channel Blockers Restore CAP Amplitude After Acrolein-Mediated Injury

Application of K^+ channel blockers significantly increased the CAP amplitude following acrolein-mediated injury. After exposure of acrolein, K^+ channel blockers, either 100 μM 4-AP or 100 μM 4-AP-3-MeOH, were perfused on the injured cord segment. CAP waveforms exhibited a striking enhancement of amplitude with both blockers, however, to a greater extent with 4-AP-3-MeOH (Fig. 3*A*). Quantification of CAP amplitude as a percentage of predrug amplitude is displayed in Fig. *3B*. Application of 4-AP-3-MeOH enhanced CAP amplitude to 209.2 ± 9.4% ($P < 0.001$, $n = 10$) and 4-AP to 180.9 ± 9.0% ($P < 0.005$, $n = 10$). In addition, the increase in amplitude with 4-AP-3-MeOH treatment was significantly greater than 4-AP ($P < 0.05$, $n = 10$). Thus it appears that, at 100 μM, 4-AP-3-MeOH is capable of restoring conduction more effectively than 4-AP. In addition to 100 μM, we have also examined the ability of 4-AP-3-MeOH to restore conduction at 0.1, 1, 10 μM, and 1 mM (Fig. 4). It is clear that while 100 μM is the most effective dosage we tested, 4-AP-3-MeOH can also effectively augment CAP conduction in acrolein-damaged axons at 0.1, 1, and 10 μM.

K^+ Channel Blockers Do Not Alter Axonal Activation Threshold

Given that both K^+ channel blockers enhanced axonal conduction following acrolein-mediated injury, their effects on axonal activation threshold were examined. Stimulation of spinal cord tissue with varying intensities ranging from 1.85 to 6.5 volts provides an assessment of the activation threshold both predrug and after K^+ channel blocker application. As demonstrated in Fig. 5, CAP amplitude increases over the entirety of the stimulating range, however, rapidly over smaller stimuli (1.85–2.5 V). In the presence of 100 μM 4-AP, the CAP amplitude is proportionally higher than predrug in the larger stimulus range tested (2.5 and 6.5 V) ($P < 0.05$, $n = 5$) (Fig. 5, *A* and *B*). The linear correlation of CAP amplitude, measured from pre-4-AP and 4-AP-treated tissue in Fig. 6*A* with a slope near unity, suggests no obvious difference in activation threshold among small- and large-caliber axons. This implies that 4-AP has no preference between large- or smaller-caliber axons when restoring axonal conduction. The CAP amplitude was normalized as a percentage of maximal amplitude with either predrug or 4-AP treatment.

Similar to 4-AP, we saw no marked change in activation threshold with 4-AP-3-MeOH treatment. Stimuli ranging from 1.85 to 6.5 volts were again applied to ventral spinal cord white matter segments before and during treatment with 100 μM 4-AP-3-MeOH. A significant increase in CAP amplitude was observed during the application of 4-AP-3-MeOH over the stimulation range of 2.5 to 6.5 volts (Fig. 5, *C* and *D*). Although this increase was greater with 4-AP-3-MeOH than 4-AP, the linear relationship in Fig. 6*B* demonstrated that again there was near unity slope of the fitting line. Therefore, little preference was observed regarding the susceptibility of axonal conduction enhancement resulting from 4-AP-3-MeOH treatment to small and large axons.

Refractory Periods Change with 4-AP but not 4-AP-3-MeOH

Multiple stimuli were applied to spinal cord tissue to assess any alteration of the refractory periods. The tissue was stimulated with dual signals of varying interstimulus time intervals from 0.5 to 13 ms to determine both the absolute and relative refractory periods. The relationship between time interval and CAP amplitude is exhibited by representative superimposed CAP waveforms as shown in Fig. 7*A*. The time interval

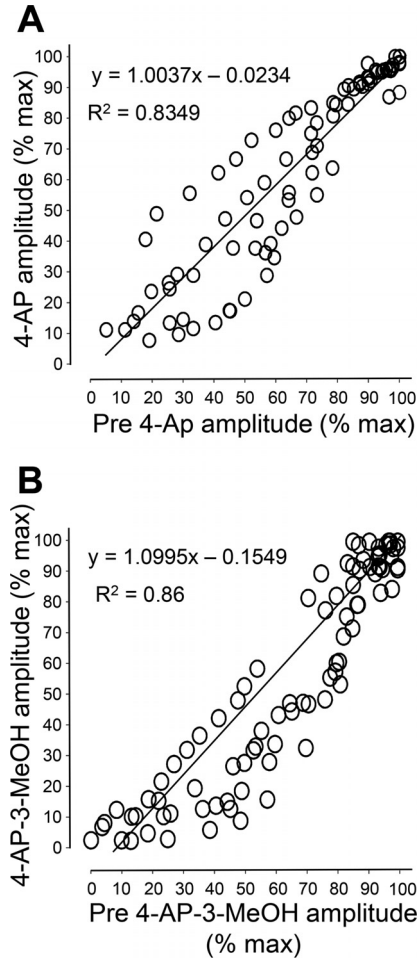

Fig. 6. Plots show the relationship between CAP amplitude predrug and following application of 4-AP or 4-AP-3-MeOH. Both 4-AP and 4-AP-3-MeOH were applied at a concentration of 100 μM. Normalized CAP responses (as %maximum CAP amplitude) of acrolein-injured spinal cord are plotted before and after the treatment of 4-AP (*A*) or before 4-AP-3-MeOH (*B*) at the same stimulus intensities for five cord samples. Each data point demonstrates the CAP response over a range of stimulus intensities (1.85–6.5 V) applied to ventral white matter spinal cord segments (same data as in Figs. 3 and 4). The overall trends of both *A* and *B* indicate a relative linear relation between predrug and the 4-AP (*A*)- or 4-AP-3-MeOH (*B*)-treated group. These data suggest a negligible difference in axon activation threshold after 4-AP or 4-AP-3-MeOH treatment in acrolein-damaged spinal cord tissue, indicating little noticeable difference in susceptibility of axons with different caliber to 4-AP- or 4-AP-3-MeOH-mediated conduction restoration.

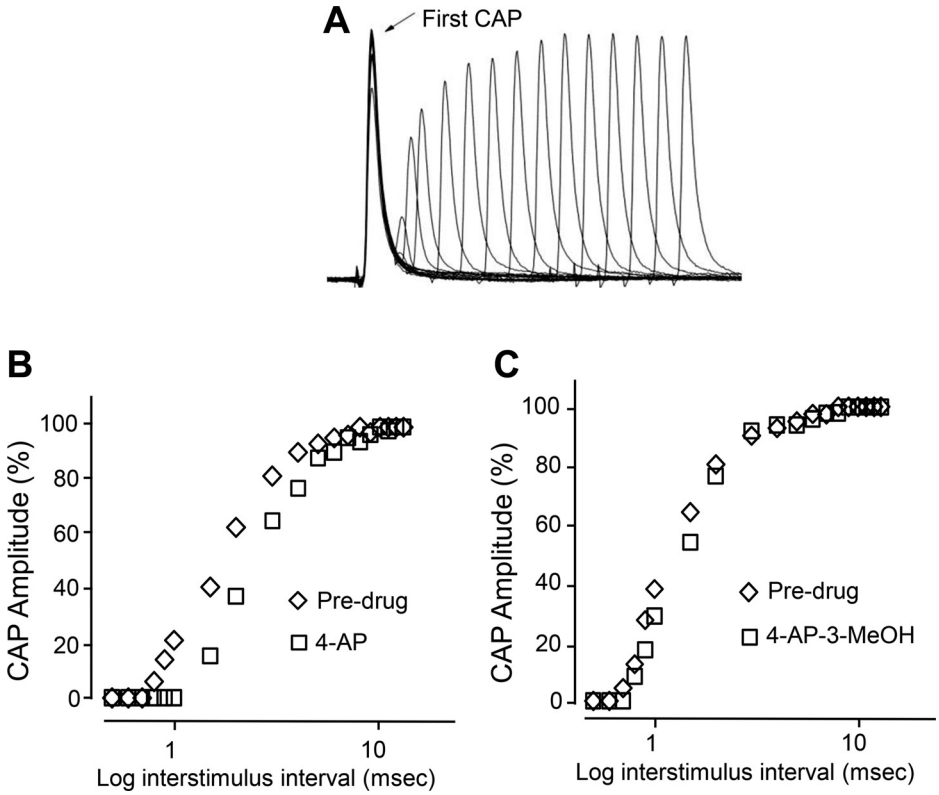

Fig. 7. Refractory period responses following the application of 4-AP (*B*) or 4-AP-3-MeOH (*C*) to acrolein-damaged spinal cord white matter segments. Refractory periods were determined using paired stimuli with varying interstimulus intervals. *A*: superimposed CAP waveforms of paired stimuli with varying interstimulus intervals. Note that the amplitude of the second CAP progressively increases due to a continuous increase in the interstimulus interval. Data in *B* and *C* present the second CAP amplitude normalized as a percentage of the first, and plotted with the log interstimulus interval in predrug and 100 μM 4-AP (*B*) or 100 μM 4-AP-3-MeOH (*C*). Note the increase of both the absolute refractory period and the relative refractory period with exposure of 4-AP, whereas no change was observed from 4-AP-3-MeOH compared with predrug.

between the first and second CAP waveform is determined as the absolute refractory period. From the same series of stimulation, the relative refractory period is established by the time interval necessary for CAP amplitude to reach 95% of the first. Compared with predrug, 100 μM 4-AP treatment caused a lengthening interstimulus time interval (Fig. 7*B*). Specifically, an increase was observed with both the absolute refractory period, from 0.72 $\pm$ 0.04 to 1.32 $\pm$ 0.23 ms ($P < 0.05$, $n = 5$), and the relative refractory period, from 4.2 $\pm$ 0.2 to 5.6 $\pm$ 0.4 ms ($P < 0.01$, $n = 5$), compared with predrug assessment (Fig. 8*A*).

With the use of the same method, 4-AP-3-MeOH application to the spinal cord was also assessed for its effects on the refractory periods. In contrast to 4-AP, little difference was observed in absolute and relative refractory periods with 4-AP-3-MeOH treatment compared with predrug recordings (Fig. 7*C*). Quantification of the absolute refractory period shows no significant change between predrug (0.9 $\pm$ 0.15 ms) and 4-AP-3-MeOH (0.96 $\pm$ 0.14 ms). The same result was observed with the relative refractory period: predrug (4.4 $\pm$ 0.75 ms) and 4-AP-3-MeOH (4.6 $\pm$ 0.68 ms) (Fig. 8*B*). Therefore, 4-AP-3-MeOH at 100 μM maintains the same absolute and relative refractory periods while restoring axonal conduction of the injured nerve fibers.

Response to Multiple Stimuli with 4-AP-3-MeOH is Consistent with Predrug

Following acrolein-mediated injury, spinal cord tissue was evaluated for its ability to maintain rapid firing with multiple stimuli in the presence of either K^+ channel blocker. Multiple stimuli were applied at low- and high-frequency stimulation, corresponding with 500 and 1,000 Hz, for 100 ms each. A representative trace of low-frequency stimulation at 500 Hz is depicted in Fig. 9*A*. Application of 100 μM 4-AP during low-frequency stimulation caused a significant reduction of the CAP amplitude, as a percentage of the first CAP amplitude, compared with predrug assessment (Fig. 9*B*). An average of the last four CAP peak amplitude, as a percentage of the first, demonstrated that low-frequency stimulation caused a drop in amplitude from 59.5 $\pm$ 3.6% predrug to 42.5 $\pm$ 7.7% with 4-AP ($P < 0.05$, $n = 5$) when quantified at the end of the train stimulation. This same reduction was also observed during high-frequency stimulation given 28.6 $\pm$ 5.5% for predrug and 18.1 $\pm$ 3.3% with 4-AP ($P < 0.05$, $n = 5$) (Fig. 10*A*).

Assessment of spinal cord tissue response to multiple stimuli with 4-AP-3-MeOH application demonstrated a different result than 4-AP. Stimulation of tissue at both low and high frequencies for 100 ms caused little alteration of the CAP amplitude in the presence of 4-AP-3-MeOH (Fig. 9*C*). No significant difference was observed with low stimuli, 57.7 $\pm$ 11.4% predrug and 56.6 $\pm$ 13.8% with 4-AP-3-MeOH, and high stimuli, 28.2 $\pm$ 9.0% predrug and 29.7 $\pm$ 9.6% with 4-AP-3-MeOH (Fig. 10*B*). From these results it appears that 4-AP-3-MeOH does not alter CAP responsiveness to repetitive stimuli at either low and high frequencies while enhancing the electrical pulse conduction of the injured axons.

DISCUSSION

In this study, we have shown that administration of the K^+ channel blocker 4-AP-3-MeOH at a concentration of 100 μM to an ex vivo rat spinal cord offers significant AP conduction restoration following myelin damage inflicted by acrolein exposure, a form of chemically induced myelin damage. Specifically, 4-AP-3-MeOH increased the CAP amplitude by >100%, which is significantly greater than that offered by the well-established K^+ channel blocker 4-AP at the same concen-

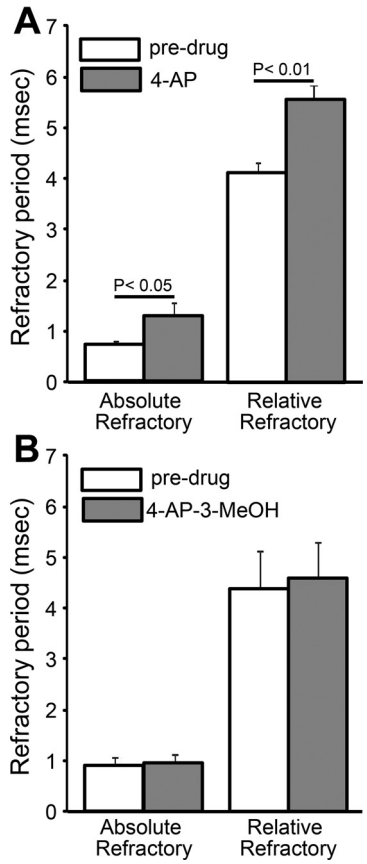

Fig. 8. Bar graph representation of absolute and relative refractory responses of predrug and with 4-AP (*A*) or 4-AP-3-MeOH (*B*) treatment in acrolein-damaged spinal cord white matter segments. The absolute refractory period is defined by the time interval necessary to elicit a second CAP, whereas the relative refractory period is defined by the time interval required for the second CAP amplitude to reach ≥95% of the first. Note that the application of 4-AP significantly increased both the absolute ($P < 0.05$, $n = 5$) and relative ($P < 0.01$, $n = 5$) refractory periods in ventral white matter spinal cord segments (*A*). In contrast, no significant change was detected in either absolute or relative refractory periods as a result of 4-AP-3-MeOH treatment. Error bars represent SE.

tration and with the same type of injuries. Therefore, in addition to AP conduction restoration capabilities in mechanically injured axons, the current data suggest that 4-AP-3-MeOH can also restore axonal conduction in those that are chemically injured. Because 100 μM is the concentration at which both 4-AP-3-MeOH and 4-AP can achieve maximal CAP amplitude increase when applied to mechanically injured spinal cord axons (Shi et al. 1997; Sun et al. 2010), we assume that 100 μM is likely the most effective dosage for both compounds in treating axons with chemically induced demyelination as well. As such, it appears that 4-AP-3-MeOH is more effective than 4-AP at reviving compromised axonal conduction due to chemical insults.

In prior studies involving mechanically injured stretched axons, we showed that 4-AP at the concentration of 100 μM can offer 50% to more than a 100% increase of CAP amplitude (Jensen and Shi 2003; Shi and Blight 1996), while 4-AP-3-MeOH can offer a 20% increase (Sun et al. 2010). Therefore, this seems to suggest that 4-AP is more effective in enhancing CAP conduction than 4-AP-3-MeOH in acute mechanically injured spinal cord axons. However, this phenomena is reversed in axons with chemically induced myelin damage as shown in the current study. In other related studies of acute (ex vivo) and chronic (in vivo) axon compression injury, 4-AP also seems to produce more robust CAP enhancement in the acute rather than chronic compression injury (Jensen and Shi 2003; Shi and Blight 1996; 1997; Shi et al. 1997). Taken together, it appears that 4-AP is more effective at restoring axonal conduction following acute mechanical injury where physical trauma is the dominate cause. However, in the chronic stage following axonal mechanical injury where secondary injury is likely to play an additional and dominate role, CAP restoration from 4-AP treatment is less robust when compared with its effects on the acute stage (Shi et al. 1997). This observation is also consistent with the current findings in this study that suggest, while 4-AP can significantly enhance CAP conduction, it is less effective than 4-AP-3-MeOH when axons are chemically injured by acrolein, a form of secondary injury characterized in neurotrauma and chronic neurodegenerative diseases.

Because 4-AP-3-MeOH is more effective in restoring CAP conductance in acrolein-induced myelin damage than when inflicted by mechanical forces, this seems to indicate that 4-AP-3-MeOH is likely more effective in restoring axonal conductance in neurodegenerative diseases, where biochemical factors are the dominant causality of myelin damage. This assumption is in good agreement with our findings that 4-AP-3-MeOH significantly increases impulse conductance in demyelinated axons isolated from EAE mice (Leung et al. 2011a). Based on the available data, it is reasonable to speculate that 4-AP-3-MeOH could potentially provide similar effective functional restoration of axons as 4-AP in MS. However, such assumption requires rigorous clinical testing and experimental comparison to 4-AP, which is currently the only available FDA-approved medication for improving ambulatory function in MS patients (Blight et al. 2014).

It is well-established that the maximal safe human serum concentration of 4-AP is no greater than 1 μM (Halter et al. 2000). Therefore, the effectiveness of 4-AP and potentially 4-AP-3-MeOH at the concentration of 100 μM, although offering the best functional recovery in ex vivo animal experimentation, is not realistic and meaningful when considered in human application. Instead, the beneficial effect of these K^+ channel blockers at concentrations near 1 μM is achievable and therefore relevant to clinical application. Based on the investigations of physical trauma where myelin damage is clearly present, the lowest effective dosage to induce significant impulse conduction enhancement is between 0.1 and 1 μM for 4-AP and 0.01 and 0.1 μM for 4-AP-3-MeOH (Jensen and Shi 2003; Sun et al. 2010). This indicates that 4-AP-3-MeOH possesses an achievable wider therapeutic range than 4-AP for the purpose of restoring axonal conductance in mechanically injured nerves, including those associated with spinal cord injury (SCI) victims. Although a dose-response curve was not conducted in this study, we believe that a similar higher potency of 4-AP-3-MeOH may still exist in chemically induced myelin damage and equivalently in MS. Indeed, 4-AP-3-MeOH at concentrations between 0.01 and 0.1 μM may be effective in restoring function in chemically induced myelin damage, a hypothesis requiring further validation in both animal and human studies.

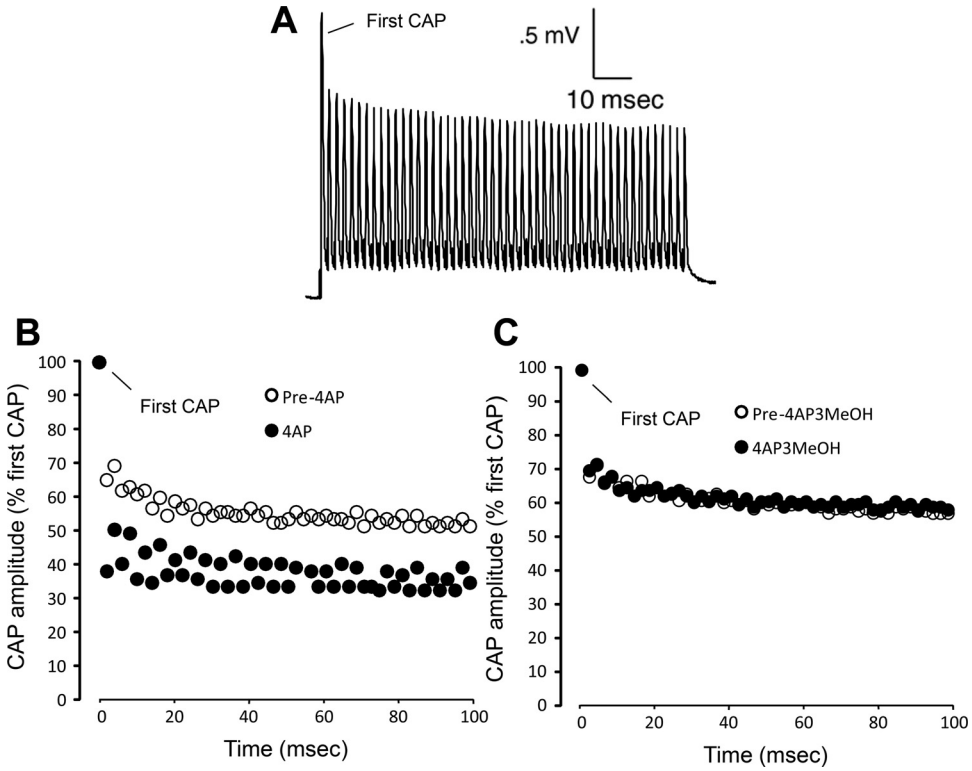

Fig. 9. The CAP response of acrolein-injured spinal cord white matter to train stimuli with and without the application of K^+ channel blockers. *A*: representative trace of CAP waveforms from typical spinal cord white matter in response to a train stimuli at 500 Hz for 100 ms. The graphs show the CAP peaks, normalized as a percentage of the first CAP, plotted against the duration of the train test (100 ms) before and after the treatment with 100 μM 4-AP (*B*) and 100 μM 4-AP-3-MeOH (*C*). A noticeable decrease in CAP amplitude was observed with 4-AP exposure. No change was observed with the treatment of 4-AP-3-MeOH.

In addition to the conductance enhancement offered by 4-AP and 4-AP-3-MeOH treatment, we also noted some similarities and differences in the electrophysiological properties in axons rescued by these two K^+ channel blockers. Consistent with previous studies, both blockers showed no preference for axon caliber when restoring AP conduction, suggesting that they rescue axons of all sizes, both large and small (Fig. 5) (Jensen and Shi 2003; Sun et al. 2010). Instead the difference is noted in the ability to follow multiple stimuli among acrolein-injured axons rescued by 4-AP or 4-AP-3-MeOH, which is similar to the result in mechanically injured axons (Jensen and Shi 2003; Sun et al. 2010). Treatment with 4-AP-3-MeOH rescues axons without affecting the property of either the absolute and relative refractory periods or repetitive firing, whereas 4-AP application led to an increase of both the relative and absolute refractory periods and the reduction of axonal capabilities to follow multiple stimuli. It has been shown that mechanical injury to the spinal cords alone caused little change in relative and absolute refractory periods of the functional surviving axonal (Shi and Borgens 1999). Therefore, it appears that injured axons with reestablished function from 4-AP-3-MeOH treatment can conduct APs in a manner closer to normal axons while 4-AP rescued axons unable to do so. As such, we postulate that 4-AP-3-MeOH may be a viable, if not preferable, alternative to 4-AP in treating axonal function losses, particularly those associated with chronic neurodegenerative diseases such as MS.

In conjunction with prior studies, the current study demonstrates that significant CAP amplitude depression occurs after ~30 min of acrolein exposure, which can be attributed in part to myelin damage due to the partial recovery observed from K^+ channel blocker treatment (Hamann et al. 2008b; Shi et al. 2011b) (Figs. 1 and 2). While this immediate functional consequence of chemically mediated damage is clear, to visualize significant structural myelin damage, such as myelin retraction and dissolution, significantly longer incubation times are required (6–12 h) based on our experience. One possible explanation is that the initial subtle structural damage such as the breakdown of the paranodal axomyelinic junction protein complex, although difficult to discern by direct observation, may be sufficient to cause functional loss (Hamann et al. 2008b; Shi et al. 2011b, 2015). This is also consistent with, or perhaps an example of, the known notion that the electrophysiological assessment is more sensitive than structural observation when assessing axonal function.

The concentration of 500 μM acrolein used in this study is consistent with levels administered in previous studies that ranged from 1 to 500 μM (Hamann et al. 2008a, 2008b; Liu-Snyder et al. 2006a, 2006b; Luo et al. 2005; Luo and Shi 2004, 2005; Shi et al. 2002, 2011b; Shi and Sun 2011). While the exact level of acrolein during trauma or disease has not been firmly established, we believe it to be within the micromolar to millimolar range. This assumption is made based on previous studies where acrolein has been estimated at elevated levels around 500 μM in the brain of Alzheimer's diseases patients (Hamann et al. 2008b; Lovell et al. 2001), 180 μM in the plasma of patients suffering renal failure (Sakata et al. 2003), and 80 μM in the respiratory fluids of smokers (Nardini et al. 2002). Furthermore, due to the limitations of this ex vivo study, the duration of acrolein application is limited to hours, whereas acrolein is more than likely elevated for a longer period of time with in vivo animal trauma and disease models and in human neurodegenerative diseases, that is, hours vs. days, months, and years. As such, the concentration threshold at which acrolein can inflict significant myelin damage in vivo is likely to be significantly lower than that used in the current study (500 μM) due to the much longer exposure time.

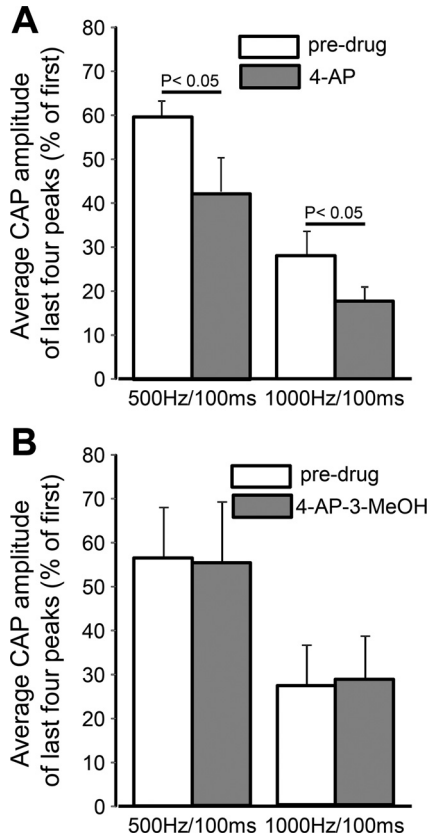

Fig. 10. Quantitative analysis of spinal cord strip CAPs in response to low (500 Hz)- and high (1,000 Hz)-frequency stimuli. Bar graphs depict the CAP responses to 500 and 1,000 Hz stimuli for a duration of 100 ms before and after treatment of 100 μM 4-AP (*A*) or 100 μM 4-AP-3-MeOH (*B*). The bar graphs show the average of the last four CAP waveforms as a percentage of the initial CAP peak. *A*: a significant reduction in CAP response was observed during 100 μM 4-AP application under both low-frequency (500 Hz; $P < 0.05$, $n = 5$) and high-frequency (1,000 Hz; $P < 0.05$, $n = 5$) stimulation. *B*: no significant changes were observed at either low (500 Hz)- and high (1,000 Hz)-frequency stimulation as a result of 4-AP-3-MeOH treatment. Error bars represent SE.

Compared with other known compounds used to produce chemically induced myelin damage, such as exogenous ethidium bromide and cuprizone compounds (Blakemore 1982; Matsushima and Morell 2001), acrolein is an endogenous chemical with strong emerging pathological roles in various animal models of trauma and neurodegenerative diseases where demyelination is implicated (Hamann and Shi 2009; Leung et al. 2011b; Park et al. 2014b; Shi et al. 2011a, 2015). In fact, acrolein has been shown to directly attack myelin in an ex vivo preparation of spinal cord and to be elevated in animal models of SCI and MS, and in addition scavenging acrolein has been shown to mitigate myelin damage and improve function (Hamann et al. 2008a, 2008b; Hamann and Shi 2009; Leung et al. 2011b; Shi et al. 2011b; Zheng et al. 2013). In light of these findings, we believe that acrolein-mediated myelin damage is a more suitable and clinically relevant model to investigate the mechanisms of myelin pathology than other artificial and exogenous myelin-damaging compounds. Furthermore, detailed mechanisms of acrolein-mediated myelin destruction are currently being elucidated (Shi et al. 2011b, 2015), which further enhances the relevance and versatility of this model to suggest other potential therapeutic targets for synergistic treatment with an anti-acrolein treatment to protect myelin such as hydralazine. For example, the neuroprotective treatment of acrolein scavenging with hydralazine can certainly be combined with the K^+ channel blockade method in efforts to not only reduce myelin damage but also restore the function of demyelinated axons (Hamann and Shi 2009; Jensen and Shi 2003; Park et al. 2014a; Shi and Sun 2011; Sun et al. 2010; Tully and Shi 2013; Tully et al. 2014). In fact, an example of this type of combination therapy has already shown not just an additive but a synergistic and potentiated effect when membrane-repairing agent polyethylene glycol was coupled with 4-AP in an ex vivo SCI model (Shi and Borgens 1999). As such, this type of combinatory treatment is more than likely a preferred strategy since most injuries and degenerative pathologies are multifaceted (Alonso et al. 2008; Compston and Coles 2008; Dumont et al. 2001; Hamann and Shi 2009; Krstic and Knuesel 2013; Przedborski 2005; Rochet et al. 2012). Even though each mechanism needs to be studied in isolation, a more successful approach will likely require a combination of treatments aiming to target multiple biochemical changes to maximize therapeutic benefits.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: R.Y. performed experiments; R.Y., J.C.P., and R.S. analyzed data; R.Y., J.C.P., and R.S. interpreted results of experiments; R.Y., J.C.P., and R.S. drafted manuscript; J.C.P. and R.S. prepared figures; J.C.P. and R.S. edited and revised manuscript; R.S. conception and design of research; R.S. approved final version of manuscript.

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