Parallel Evaluation of Two Potassium Channel Blockers in Restoring Conduction in Mechanical Spinal Cord Injury in Rat

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Abstract

Myelin damage is a hallmark of spinal cord injury (SCI), and potassium channel blocker (PCB) is proven effective to restore axonal conduction and regain neurological function. Aiming to improve this therapy beyond the U.S. Food and Drug Administration–approved 4-aminopyridine (4-AP), we have developed multiple new PCBs, with 4-aminopyridine-3-methanol (4-AP-3-MeOH) being the most potent and effective. The current study evaluated two PCBs, 4-AP-3-MeOH and 4-AP, in parallel in both *ex vivo* and *in vivo* rat mechanical SCI models. Specifically, 4-AP-3-MeOH induced significantly greater augmentation of axonal conduction than 4-AP in both acute and chronic injury. 4-AP-3-MeOH had no negative influence on the electrical responsiveness of rescued axons whereas 4-AP-recruited axons displayed a reduced ability to follow multiple stimuli. In addition, 4-AP-3-MeOH can be applied intraperitoneally at a dose that is at least 5 times higher (5 mg/kg) than that of 4-AP (1 mg/kg) *in vivo*. Further, 5 mg/kg of 4-AP-3-MeOH significantly improved motor function whereas both 4-AP-3-MeOH (1 and 5 mg/kg) and, to a lesser degree, 4-AP (1 mg/kg) alleviated neuropathic pain-like behavior when applied in rats 2 weeks post-SCI. Based on these and other findings, we conclude that 4-AP-3-MeOH appears to be more advantageous over 4-AP in restoring axonal conduction because of the combination of its higher efficacy in enhancing the amplitude of compound action potential, lesser negative effect on axonal responsiveness to multiple stimuli, and wider therapeutic range in both *ex vivo* and *in vivo* application. As a result, 4-AP-3-MeOH has emerged as a strong alternative to 4-AP that can complement the effectiveness, and even partially overcome the shortcomings, of 4-AP in the treatment of neurotrauma and degenerative diseases where myelin damage is implicated.

Keywords: 4-aminopyridine; 4-aminopyridine-3-methanol; demyelination; spinal cord injury

Introduction

THE MAMMALIAN SPINAL CORD is comprised of a myelinated axonal network essential for the transmission of electrical signals.^{1,2} The breakdown of its structural integrity attributed to spinal cord injury (SCI) results in loss of motor and sensory function.^{2–9} Despite decades of efforts, very few treatment strategies are currently available.^{10–12} As such, understanding the molecular pathological mechanisms linking axonal structure damage and conduction loss is of great importance to elucidate the post-SCI neurological deficits and establish effective treatments to restore function.^{5,6,13,14}

It is well established that axonal functional loss could stem from damage to the myelin.^{3,13,15–17} Myelin, a lipid insulator of axons, is crucial for an efficient form of axonal signal transmission, saltatory conduction.^{1,2} Unfortunately, disruption of myelin structure could have profound functional consequences, and few treatments are

currently available to effectively address this problem; that is, either to deter the myelin deterioration or to restore the function of myelin-damaged axons.^{3,5,13,15–17}

Under physiological conditions, axons traversing the spinal cord are wrapped with myelin except at the nodes of Ranvier, where voltage-dependent sodium channels (Na_v) critical for generating action potentials (APs) are clustered at axolemma.^{1,2} Initiation of AP at the nodes relies on the depolarization of nodal axonal membrane and the adequate sodium ions (Na⁺) influx through Na_v, which allows for AP generation in the next node, thereby propagating and perpetuating the AP forward.^{1,2,5} In the juxtaparanodal area, an internodal region that is close to the nodes of Ranvier, there are cluster of voltage-gated potassium channels (K_v). These K⁺ channels expressed under the myelin are thought to contribute to the maintenance of internodal resting potential.¹ It has been shown, both experimentally and mathematically, that such efficient axonal

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conductance could be compromised when myelin is damaged and K_v channels, normally located at the juxtaparanodal region, are unmasked.^{13,14,16,18–21} Specifically, the exposed K_v channels in the juxtaparanodal area shunt the electrotonic signal and prevent Na_v activation and AP propagation.^{2,6,13–15,18,21–26}

Subsequent to recognition of the key pathological role of K_v channels in axonal functional loss in those with myelin damage, potassium channel blockers (PCBs) have been examined to block K_v channels, encourage adequate depolarization of nodal axonal membrane, and ultimately restore the conduction of AP in injured axons.^{6,15,24,26–32} Such a strategy has been shown to be effective in the treatment of various demyelinated conditions in animal experiments in the last several decades.^{6,15,26–28,31–34} 4-Aminopyridine (4-AP), the most-studied PCB, has been approved by the U.S. Food and Drug Administration (FDA) for treatment of multiple sclerosis (MS) patients, a neurodegenerative disease marked by chronic myelin damage, after successful pre-clinical and clinical studies.^{15,24,29,35–37} Although beneficial, the effectiveness of 4-AP is nevertheless modest because of a narrow achievable safe and effective dosage in humans and potential side effects when plasma levels are beyond 1 μ M.^{38,39} As such, multiple new generations of PCBs have been developed aiming to broaden therapeutic range and reduce side effects.^{30,33,34,40}

Among multiple new PCBs, 4-aminopyridine-3-methanol (4-AP-3-MeOH) has emerged as the most promising choice for restoring axonal function.^{6,26,30} In the current study, we set out to examine the effect of both 4-AP and 4-AP-3-MeOH in parallel for the first time in *ex vivo* and *in vivo* mechanical SCI models. We have shown that both 4-AP and 4-AP-3-MeOH can significantly restore axonal conduction in a rat model of mechanical SCI. However, side-by-side comparison demonstrates the advantages of 4-AP-3-MeOH over 4-AP. Interestingly, 4-AP-3-MeOH can improve motor function in live animals after contusive SCI and can be applied at 5 times the concentration of 4-AP *in vivo*. Further, both compounds demonstrated the effects of transient, yet significant, reduction of post-SCI hypersensitivity. The analgesic effect of 4-AP-3-MeOH is particularly prominent and dose dependent. These data support the potential of 4-AP-3-MeOH as an effective agent for restoring axonal function post-SCI, which could mitigate not only motor, but also sensory dysfunction.

Methods

Experimental animals

All animals in this study were used under strict accordance to the Purdue University Animal Care and Use Committee protocol and guidelines. A total of 61 adult male Sprague-Dawley rats (Envigo RMS, Inc., Indianapolis, IN) ranging from 200 to 400 g were used. Animals were anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg) through intraperitoneal (i.p.) injections. Animals used for survival surgeries were acclimated to animal housing for at least 1 week pre-surgery.

Spinal cord surgical extraction

After anesthesia and sensory deprivation, a transcardial perfusion was performed with cold oxygenated (95% O_2 , 5% CO_2) Krebs solution. Rapid removal of the vertebrae column was followed by a complete laminectomy and careful excision of the spinal cord by cutting spinal roots. The dura mater was independently removed and ventral white matter strips were collected by subdividing the spinal cord twice longitudinally. Tissue segments were incubated in fresh Krebs solution for 60 min at room temperature and bubbled continuously with 95% O_2 , 5% CO_2 to maintain a pH of 7.2–7.4. Krebs solution contained (mM): 124.0

NaCl, 2.0 KCl, 1.2 KH_2PO_4 , 1.3 $MgSO_4$, 26.0 $NaHCO_3$, 5.6 sodium ascorbate, 10.0 dextrose, and 1.2 $CaCl_2$.

Double sucrose gap recordings

Spinal cord sections were placed on a double sucrose gap recording chamber for functional assessments.^{6,15,30,41} In the recording chamber, spinal cord segments were placed across the central compartment, adjacent sucrose gap compartments, and outside wells. The central compartment was continuously perfused (2 mL/min) with 37°C Krebs solution, sucrose gaps were perfused (1 mL/min) with isotonic sucrose (320 mM), and the outer wells were filled with isotonic KCl (120 mM). To keep compartments isolated, vacuum grease and microplastic plates were used to seal spinal cord tissue on both sides of the sucrose gap chambers. Ag/Cl electrodes in the central and outer KCl compartments were used to stimulate and record axonal conduction. Electrodes in the central compartment were connected to an instrument ground. Axons were stimulated with 0.1-ms constant current unipolar pulses at one end and recorded at the opposite end. All conduction occurred along the length of the central compartment. Recordings and data acquisition were accomplished using a bridge amplifier (Neuro Data Instruments, New York, NY) and custom Labview software (National Instruments, Woburn, MA) on a Dell PCTM (Dell, Round Rock, TX).

Compound action potentials

Compound action potentials (CAPs) are the spatiotemporal summation of all single-unit action potentials fired by individual axons. Assessment of CAP amplitudes was measured through constant application of 0.1-ms unipolar pulses every 3 sec with a supramaximal stimulus (110% of the maximal stimulus). Multiple CAP waveforms were captured at pre-determined times and averaged for subsequent analysis. In addition, real-time plots of the CAP amplitude through the course of the experiment were recorded and saved for future reference.^{6,15,30,41}

Activation threshold

Spinal cord segments stimulated in the double sucrose gap recording chamber were assessed for axon activation thresholds. Large-caliber axons were activated at lower voltages whereas small-caliber axons remained inactive until higher voltages were applied. Current-voltage tests were performed by applying stimuli at increasing voltages from 1.85 to 6.5 V. Each voltage was repeated five times and an average was taken for analysis. The stimulus was delivered at a frequency of one every 3 sec.

Absolute and relative refractory periods

Refractory periods were measured using twin stimuli (110% the maximal stimulus) with an increasing interstimulus interval. The interval ranged from 0.5 to 15 msec, where each stimulus interval was repeated five times and an average taken. The amplitude of the first CAP remained the same for each twin stimuli. Absolute refractory periods were defined by the time required to illicit a second CAP, whereas the relative refractory periods were defined by the time required to illicit a second CAP with an amplitude that was 90% or greater than that of the first CAP.

Frequency stimulation response

Axonal conduction response to frequency stimulation was measured over a short period of time where trains of repetitive stimuli at a supramaximal stimulus (110% of the maximal stimulus) were applied. Spinal cord segments were stimulated with low- and high-frequency stimulation at 500 and 1000 Hz for 100 ms, which elicited 50 and 100 CAPs respectively. The last three CAPs were averaged and expressed as a percentage of the first CAP. Each measurement was repeated five times and an average was taken for analysis.

Ex vivo spinal cord stretch injury model

An SCI device was used to induce a constant degree of stretch that is detailed in previous publications.^{6,15,42,43} The spinal cord strip was placed across the center compartment in the double sucrose gap recording chamber, resting on a flat raised stage with a central hole. A stretch rod released from a pre-determined height fell at ~1.5 m/s on to the cord to induce the stretch injury. To stabilize tissue through the injury procedure, a nylon mesh was laid on top of the cord in the chamber. Immediately post-injury, the rod and nylon mesh were removed.

In vivo contusive injury model

Animals were anesthetized pre-surgery as described above. The dorsal surface of the T-10 spinal cord was exposed after removal of the spinous process and vertebral lamina. The exposed surface of the spinal cord was injured with an NYU (New York University) weight-drop impactor using a 10-g rod 3 mm in diameter. Injury was generated by dropping the rod at heights of 12.5 mm (mild) or 25 mm (moderate). Sham animals received a laminectomy of T-10 vertebrae only. Post-operative care included daily manual bladder expression in contused rats until reflexive control of bladder function returned. Saline (3 mL) was administered subcutaneously to prevent dehydration.^{44–46} For the purpose of *ex vivo* evaluation, 3–6 months post-SCI, spinal cord tissue was excised and placed in the double sucrose gap recording chamber. *In vivo* evaluation, both motor and sensory behavior, were conducted 2 weeks post-injury.

Motor function behavioral assessment

Locomotion of animals was assessed using a rotarod test 2 weeks post-moderate contusive SCI. Rotarod performance test was used to assess the strength and coordination in the lower extremities known to be impaired in SCI.⁴⁷ Rats were tested on the rotarod at increasing revolutions per minute (rpm) of 0–30 rpm over 3 min and then remained at 30 rpm. They were assessed for the time on rotarod and rpm the animal could sustain before falling off. Each trial was repeated three times.

Nociception behavioral assessments

Impacts of potassium channel blockers (4-AP and 4-AP-3-MeOH) on neuropathic pain behaviors were investigated through mechanical allodynia and cold hyperalgesia tests. Specifically, a hindlimb von Frey and cold test was conducted to assess the “below-SCI-level” hypersensitivity. Either 1 or 5 mg/kg of PCBs

FIG. 1. Axonal conduction changes in response to stretch injury and K⁺ channel blockers. (A) Compound action potential (CAP) changes in response to stretch injury *ex vivo*. Upper panel: selective typical CAP waveforms showing the decrease and recovery of CAP amplitude attributed to stretch injury. Lower panel: time course of the changes of CAP amplitude demonstrating the immediate drop in CAP amplitude after a stretch injury that gradually recovered to a stable level, which is approximately 40% its original magnitude, over a 30-min time frame. (B) Partial restoration of CAP amplitude attributed to K⁺ channel blocker treatment after *ex vivo* stretch injury. Example waveforms show increased amplitude with the treatment of either 4-AP or 4-AP-3-MeOH. The bar graph depicts the effects of 100 μ M of 4-AP (***) $p < 0.001$; $N = 12$) or 100 μ M of 4-AP-3-MeOH (***) $p < 0.001$; $N = 7$) to significantly enhance CAP amplitude post-SCI, where 4-AP-3-MeOH significantly improves amplitude in comparison with 4-AP (##) $p < 0.01$). Error bars represent SEM. 4-AP, 4-aminopyridine; 4-AP-3-MeOH, 4-aminopyridine-3-methanol; SCI, spinal cord injury; SEM, standard error of the mean.

were administered by i.p. injection 2 weeks post-SCI. Behavioral tests time points were 1, 2, 3, 4, 5, 6, and 24 h after PCB injection.

Mechanical allodynia

As an indicator of mechanical hyper-reflexia, a foot withdrawal threshold to mechanical stimuli was used. Rats were placed on a metal mesh floor, covered by a transparent plastic box, and allowed to acclimate alone for 10 min before testing. Mechanical stimulation used calibrated von Frey filaments: 0.4, 0.6, 1.0, 2.0, 4.0, 6.0, 8.0, and 15.0 g (Stoelting, Wood Dale, IL). Each filament was applied 3–5 sec perpendicular to the plantar surface of the hindlimb with sufficient bending force at a frequency of one per minute. A positive response was considered rapid withdrawal of the hindlimb with or without licking and biting. When a positive response was observed, a lower-grade filament was then applied, and in the absence of a response, the next greater stimulus filament was presented. The 50% of the mechanical thresholds were calculated based on the up-down method.⁴⁸

Cold hyperalgesia

Sensitivity to cold application was measured using the 100% acetone-evoked evaporative cooling test.^{49,50} Similar to mechanical hyper-reflexia, animals were placed on a metal mesh floor, covered with a transparent plastic box, and acclimated to their surroundings for 10 min before testing. Acetone (0.05 mL) was applied 2 mm from the plantar surface of the hind paw, and withdrawal or hind paw licking response indicated cold hyperreflexia. The acetone was applied five times to each plantar paw at intervals of 5 min.

Pharmacological agents

4-AP (Sigma-Aldrich, St. Louis, MO) and 4-AP-3-MeOH (Matrix Scientific, Elgin, SC) were used in all experiments. For spinal cord segments, each compound was first dissolved into ddH₂O to make a stock solution of 10 mM. Before use for recordings, each compound was diluted to desirable concentration in normal Krebs solution.^{29,30} Solutions were continuously oxygenated and maintained at 37°C.

For behavioral assessments after acute application of PCBs, both 4-AP and 4-AP-3-MeOH were dissolved in sterilized phosphate-buffered saline at a concentration of 1 mg/mL (pH at 7.4). The final injection volume was based on the weight of the rats, aiming to reach a final concentration of 1 or 5 mg/kg. Therefore, for a rat with a body weight of 250 g, 1.25 mL was needed for a dosage of 5 mg/kg, and 0.25 mL was needed to reach a dosage of 1 mg/kg. Each compound was applied through i.p. injections 2 weeks post-contusive SCI.

Statistical analysis

For comparison of electrophysiological recordings, a paired Student's *t*-test was used. The Pearson correlation coefficient (*r*) was used to evaluate the linear correlation of some electrophysiology measurements. One-way analysis of variance and Tukey's honestly significant difference were used to detect significance

between groups for behavioral motor and pain assessments. Statistical significance was measured at $p < 0.05$, and averages are shown as mean $\pm$ standard error of the mean (SEM).

Results

4-AP-3-MeOH induced greater augmentation of CAP amplitude after stretch than 4-AP in isolated spinal cord segment

The cord segment was first placed on the double sucrose gap, and the CAP was monitored until amplitude stabilized for at least 10 min. The cord was then stretched, which resulted in an immediate significant reduction in CAP amplitude. Conduction gradually recovered to a steady point, as observed by the increased CAP amplitude at approximately 30 min post-injury (Fig. 1A). After stabilization of the recovery of CAP amplitude, either 4-AP or 4-AP-3-MeOH, dissolved in oxygenated Krebs solution, were continuously perfused onto the cord for an average of 45 min.

CAP amplitude of stretched cords significantly increased with application of either 100 μ M of 4-AP or 100 μ M of 4-AP-3-MeOH, as shown in Figure 1B. Specifically, the increase of CAP amplitude compared to pre-drug (normalized as 100%) attributed to 100 μ M of 4-AP was $121.59 \pm 2.0\%$ ($p < 0.001$; $N = 12$), and the augmentation of CAP amplitude resulting from 100 μ M of 4-AP-3-MeOH was $135.51 \pm 1.8\%$ ($p < 0.001$; $N = 7$). Further, CAP amplitude increases caused by 4-AP-3-MeOH were significantly greater than those resulting from 4-AP ($p < 0.01$).

Axonal activation threshold remains unaffected by 4-AP-3-MeOH and 4-AP

To assess and compare the effects of 4-AP or 4-AP-3-MeOH on the activation threshold of recruited axons after stretch *ex vivo*, stimuli ranging in intensities from 1.85 to 6.5 V were applied to injured spinal cord tissue pre- and post-treatment. Original waveforms of CAP in response to increasing stimulus intensities showed an overall increase in CAP amplitude from pre-drug to post-treatment of either 100 μ M of 4-AP or 100 μ M of 4-AP-3-MeOH (Fig. 2A). Treatment with either compound did not significantly change the relative amplitude-stimulus intensity curves when compared with pre-drug (Fig. 2B,C). Additional comparison of CAP amplitudes pre- and post-drug with a linear correlation produced slopes close to 1 (Fig. 2D,E), indicating minimal bias based on axon caliber and activation threshold. It was clear that although changes in amplitude were present, they were not a result of change in activation threshold.

4-AP-3-MeOH-recruited axons maintain normal response to dual and multiple stimuli

To assess the refractory periods of newly recruited axons from treatment with PCBs, post-SCI dual stimuli were applied at a 110%

FIG. 2. Assessment of activation threshold changes after 4-AP or 4-AP-3-MeOH application in injured spinal cord. (A) Spinal cord ventral white matter segments were stimulated with intensities ranging from 1.85 to 6.5 V, and the responding compound action potential (CAP) waveforms were superimposed to demonstrate the amplitude changes in both pre-drug and post-treatment (4-AP or 4-AP-3-MeOH) conditions. (B,C) Plots show normalized CAP responses (% of max CAP amplitude pre-drug) of injured spinal tissue at each stimuli intensity. Note a significant increase in CAP amplitude from pre-drug to 100 μ M of 4-AP ($N = 10$; B) or 100 μ M of 4-AP-3-MeOH ($N = 5$; C) application at stronger stimuli intensities. (D,E) Plots show the relationship between normalized CAP amplitudes of pre-drug and after the treatment of 4-AP (D) or 4-AP-3-MeOH (E) are plotted at the same stimulus intensities. The relative linear relations suggest minimal changes in activation threshold after either 4-AP or 4-AP-3-MeOH application to injured spinal cord segment *ex vivo*. This also indicates little difference in the susceptibility of axons with small or large caliber to the 4-AP- or 4-AP-3-MeOH-mediated conduction restoration. 4-AP, 4-aminopyridine; 4-AP-3-MeOH, 4-aminopyridine-3-methanol.

supramaximal stimulus with increasing interstimulus intervals from 0.5 to 15 ms. Figure 3A depicts an example of a series of superimposed dual CAP waveforms across the entire interstimulus range. During each twin stimuli application, the first stimuli generated a CAP waveform with a relatively constant amplitude

whereas the second stimuli and resulting CAP waveform (overlaid) required longer time intervals to attain a similar amplitude. Amplitudes of the second CAP of both pre- and post-drug are shown in Figure 3B (for 4-AP) and 3C (for 4-AP-3-MeOH). Quantification of the absolute and relative refractory periods of pre- and post-drug for

both 4-AP and 4-AP-3-MeOH were calculated, averaged, and compared as shown in Figure 3D,E.

It appeared that 4-AP-recruited axons required longer interstimulus intervals to restore the amplitude of the second CAP in the dual stimuli paradigm, whereas such a tendency was much less obvious, if any, in 4-AP-3-MeOH-treated axons (Fig. 3B,C). We defined the absolute refractory period as the time required to elicit a second CAP and relative refractory as the time sufficient to elicit a second CAP with a peak amplitude $\geq 90\%$ of the first CAP. Our results show that $100\ \mu\text{M}$ of 4-AP caused a significant increase in both absolute refractory (pre-drug, 1.3 ± 0.001 ms; 4-AP, 1.7 ± 0.001 ms; $p < 0.01$; $N=9$) and relative refractory (pre-drug, 5.6 ± 0.002 ms; 4-AP, 8.1 ± 0.003 ms; $p < 0.001$; $N=9$) periods (Fig. 3D). In contrast, $100\ \mu\text{M}$ of 4-AP-3-MeOH did not significantly affect absolute (pre-drug, 1.3 ± 0.001 ms; 4-AP-3-MeOH, 1.4 ± 0.001 ms; $p > 0.05$; $N=7$) or relative refractory (pre-drug, 5.5 ± 0.002 ms; 4-AP-3-MeOH, 5.5 ± 0.002 ms; $p > 0.05$; $N=7$) periods (Fig. 3E).

Axons recruited by 4-AP or 4-AP-3-MeOH after stretch injuries were also tested for their ability to respond to multiple stimuli. A low- (500 Hz) and high-frequency (1000 Hz) train stimulus was applied over 100 ms to injured spinal cord tissue pre- and post-drug. Figure 4A shows a representative trace of low-frequency stimulation 30 min after stretch SCI. Recordings were quantified by averaging the final three CAP amplitudes as a percentage of the first CAP. Figure 4B shows that the treatment of $100\ \mu\text{M}$ of 4-AP resulted in a significant decrease in CAP amplitude of the last three CAPs in the low-frequency stimulation (pre-drug, $33.9 \pm 3.7\%$; 4-AP, $19.4 \pm 4.0\%$; $p < 0.05$; $N=5$) and a similar decrease of CAP amplitude in the high-frequency stimulation (pre-drug, $15.8 \pm 2.2\%$; 4-AP, $10.3 \pm 1.9\%$; $p < 0.05$; $N=5$). In contrast, $100\ \mu\text{M}$ of 4-AP-3-MeOH did not cause CAP reduction in low-frequency stimulation (pre-drug, $34.2 \pm 3.6\%$; 4-AP-3-MeOH, $34.7 \pm 3.7\%$; $P > 0.05$; $N=6$) and high-frequency stimulation (pre-drug, $14.7 \pm 1.6\%$; 4-AP-3-MeOH, $14.8 \pm 2.7\%$; $p > 0.05$; $N=3$; Fig. 4C).

4-AP and 4-AP-3-MeOH augment compound action potential amplitude after *in vivo* contusive spinal cord injury

The effect of 4-AP and 4-AP-3-MeOH to restore axonal conduction was further compared after an *in vivo* contusive SCI model, and conduction was assessed using double sucrose gap recordings on excised cord segments. After a contusive SCI, 4-AP and 4-AP-3-MeOH, both at $100\ \mu\text{M}$, can produce a significant increase in CAP amplitude ($118.7 \pm 4.0\%$; $p < 0.05$; $N=4$; for 4-AP, and $148.2 \pm 6.9\%$; $p < 0.01$; $N=5$ for 4-AP-3-MeOH). Further, augmentation of CAP amplitude from 4-AP-3-MeOH was greater than that caused by 4-AP ($p < 0.01$; Fig. 5A).

Enhancement of motor behavioral function by 4-AP-3-MeOH and analgesic effect provided by both 4-AP and 4-AP-3-MeOH

The ability to enhance motor function in live animals suffering a SCI was accomplished through the treatment of either 4-AP or 4-AP-3-MeOH, using a rat contusive SCI model. Each compound was administered 2 weeks post-SCI through an i.p. injection at one of two doses: 1 or 5 mg/kg for 4-AP-3-MeOH and 1 mg/kg for 4-AP. The 5-mg/kg dosage was not adopted for 4-AP because of the fact that treated animals exhibited debilitating seizures and were excluded from the study (data not shown). Animals were then placed on a rotarod and assessed for motor coordination based on both the maximal time(s) on rotarod and rpm that the rat could achieve. Each animal was tested three times and an average was taken for quantitative analysis. Uninjured animals served as a control. At a treatment dosage of 5 mg/kg, 4-AP-3-MeOH significantly improved time on the rotarod ($p < 0.05$; $N=5$) and rpm ($p < 0.05$; $N=5$; Fig. 6A,B). However, at a dosage of 1 mg/kg, no significant changes were observed with the treatment of either 4-AP or 4-AP-3-MeOH.

Both 4-AP and 4-AP-3-MeOH were tested for their ability to provide relief on post-SCI hypersensitivity in the rat. Rat neuropathic pain behavior was assessed using von Frey filament assay (for mechanical allodynia) and acetone spray assay for cold hyperalgesia. Assessments were conducted for the first 6 h and again at 24 h after one bolus injection of PCB. As indicated in Figure 7, a significant reduction in von Frey mechanical hypersensitivity was observed for: 1 mg/kg 4-AP (peak pain relief at 2 h post-injection), 1 mg/kg of 4-AP-3-MeOH (maximum pain relief at 3–4 h post-injection), and, most notably, 5 mg/kg of 4-AP-3-MeOH (maximum pain relief at 6 h post-injection; Fig. 7A). A similar result was observed with thermal hyperalgesia where a significant reduction in paw withdrawal frequency was observed for: 1 mg/kg of 4-AP, 1 mg/kg of 4-AP-3-MeOH, and, most notably, 5 mg/kg of 4-AP-3-MeOH (Fig. 7B).

Discussion

In the current study, we have shown that in a rat spinal cord mechanical injury model, both 4-AP and 4-AP-3-MeOH can augment the amplitude of CAPs when examined in parallel, where 4-AP-3-MeOH demonstrated a more significant CAP enhancement compared to that elicited by 4-AP (Figs. 2 and 6). In addition, 4-AP-3-MeOH at 5 mg/kg induced a significant improvement of motor function post-SCI based on rotarod assessment, whereas both 4-AP and 4-AP-3-MeOH failed to do so at 1 mg/kg, and 4-AP at 5 mg/kg produced seizure-like behavior (Fig. 6). Finally, whereas both 4-AP and 4-AP-3-MeOH mitigated post-SCI hypersensitivity at 1 mg/kg, 4-AP-3-MeOH at 5 mg/kg provided the most significant analgesic effect (Fig. 7). These data indicate that not only has 4-AP-4-MeOH

FIG. 3. Absolute and relative refractory period changes after treatment of 4-AP or 4-AP-3-MeOH in injured spinal cord segments. (A) The superimposed individual compound action potential (CAP) waveforms depict the response of ventral white matter to dual stimuli with increasing interstimuli intervals. The constant stimulus intensity has led to the initial CAP peaking at a consistent amplitude. However, as the interstimulus interval progresses, the amplitude of the second CAP of each recording changes. (B,C) The amplitude of the second CAP, as a percentage of the first, is plotted as a function of the log interstimulus interval for both pre-drug and after $100\ \mu\text{M}$ of 4-AP (B) or $100\ \mu\text{M}$ of 4-AP-3-MeOH (C) treatment. (D,E) Bar graph showing the changes of absolute and relative refractory periods as a result of 4-AP or 4-AP-3-MeOH application. The interstimulus interval at which the second responding CAP first appeared is defined as the absolute refractory period, whereas the time interval required for the amplitude of the second CAP to reach 90% of the first one is defined as the relative refractory period. After treatment with 4-AP, a significant increase is observed in both the absolute ($**p < 0.01$; $N=9$) and relative ($***p < 0.001$) refractory periods (D). No significant changes were detected in both types of refractory periods when treated with 4-AP-3-MeOH ($N=7$; E). Error bars represent SEM. 4-AP, 4-aminopyridine; 4-AP-3-MeOH, 4-aminopyridine-3-methanol; SEM, standard error of the mean.

FIG. 4. Compound action potential (CAP) response of injured white matter segment to train stimuli pre- and post- K^+ channel blocker application. Typical CAP waveforms from spinal cord white matter in response to a train stimuli at 500 Hz for 100 ms is shown. (A,B) Bar graph demonstrates the quantitative analysis of the CAP response to train stimuli pre-drug compared with the treatment of 100 μ M of 4-AP (A) or 100 μ M of 4-AP-3-MeOH (B) in injured spinal cord white matter. Train stimuli (100 ms) with low (500 Hz) or high (1000 Hz) frequency was applied to injured spinal cord strips pre-drug and after 4-AP or 4-AP-3-MeOH. Data show the averaged amplitudes of the last three CAPs as a percentage of the first CAP. 4-AP treatment resulted in a significant decrease in CAP amplitude in response to train stimuli at both the low frequency (* $p < 0.01$; $N = 5$) and high frequency (* $p < 0.05$; $N = 5$). Conversely, with the treatment of 4-AP-3-MeOH, there were no significant differences in CAP response at either the low frequency ($N = 6$) or high frequency ($N = 3$). Error bars represent SEM. 4-AP, 4-aminopyridine; 4-AP-3-MeOH, 4-aminopyridine-3-methanol; SEM, standard error of the mean.

the ability to restore axonal conduction in mechanically injured axons, but it does so more effectively than 4-AP. Considering in a previous study involving chemical (acrolein)-mediated axonal injury where 4-AP-3-MeOH also displayed a superior capability over 4-AP in restoring axonal conduction,⁶ we conclude that 4-AP-3-MeOH has emerged as a strong alternative, if not better, PCB in axonal functional restoration with myelin damage attributed to both mechanical and chemical causalities, both known pathological contributors of mammalian SCI.

4-AP is a well-established PCB that has been known for decades for its ability to restore axonal conduction in injured, yet anatomically continuous, axons with myelin damage.^{6,15,24,26–29,32,37–39} In contrast, 4-AP-3-MeOH has a considerably shorter history of being known as a PCB to restore axonal conduction, which was first reported by our group in 2010.³⁰ Subsequently, 4-AP-3-MeOH has been demonstrated to enhance conduction in multiple injury models and in various preparations.^{6,26,30,31} Based on available

evidence, 4-AP-3-MeOH has demonstrated its advantages over 4-AP in multiple aspects in addition to being more effective in the augmentation of CAP amplitude.

First, the axons rescued by 4-AP-3-MeOH behaved in a manner similar to normal axons when responding to multiple stimuli whereas those reinstated by 4-AP displayed a compromised ability to do so (Figs. 3 and 4).^{6,15,30} This is consistent with the findings that 4-AP rescued axons have a significantly longer refractory period when compared to those rescued by 4-AP-3-MeOH, although the mechanisms of such difference have not been investigated yet. Second, whereas 4-AP at 5 mg/kg showed toxicity of causing seizer-like behavior, 4-AP-3-MeOH at such a concentration was safe and provided motor behavioral benefits *in vivo* post-SCI (Fig. 6). Third, the threshold of 4-AP-3-MeOH that can cause CAP enhancement in axons with myelin damage (between 0.01 and 0.1 μ M) is 10 times less than that of 4-AP (between 0.1 and 1 μ M). This indicates that 4-AP-3-MeOH is more potent and has a wider

FIG. 5. Augmentation of compound action potential (CAP) amplitude resulted from treatment of K⁺ channel blocker after *in vivo* mild contusive SCI. Example waveforms show increased CAP amplitude when treated with 4-AP or 4-AP-3-MeOH. The bar graph depicts the effects of 100 μ M of 4-AP (* p < 0.05; N = 4) or 100 μ M of 4-AP-3-MeOH (** p < 0.01; N = 5) to significantly enhance CAP amplitudes post-mild SCI. Treatment with 4-AP-3-MeOH also resulted in a significantly greater increase of CAP amplitude when compared to treatment with 4-AP (** p < 0.01). Error bars represent SEM. 4-AP, 4-aminopyridine; 4-AP-3-MeOH, 4-aminopyridine-3-methanol; SCI, spinal cord injury; SEM, standard error of the mean.

range in restoring axonal conduction.^{6,15,30} Fourth, 4-AP-3-MeOH has been noted for suppressing potassium channel current other than the classic transient A-type current that may be important when conduction failure is attributed to myelin damage.³⁰ For example, 4-AP-3-MeOH has been shown to effectively suppress a long-lasting potassium (I_A) current whereas 4-AP has little effect on this current, which may contribute to the superiority of 4-AP-3-MeOH in restoring CAP amplitude when compared to 4-AP.³⁰ In summary, 4-AP-3-MeOH appears to be more advantageous over 4-AP in restoring axonal conduction attributed to the combination of its higher efficacy in enhancing CAP amplitude, known ability to suppress additional potassium current, lesser negative effect on axonal responsiveness to multiple stimuli, and wider therapeutic range in both *ex vivo* and *in vivo* application.

The current finding also constitutes the first observation that 4-AP-3-MeOH has the ability to restore axonal conduction in

chronic SCI. In a previous separate study with similar methodology, 4-AP at 100 μ M was shown to induce an augmentation of CAP that is comparable to that observed in the current study using 4-AP at the same concentration (Fig. 5).²⁹ In this investigation, 4-AP-3-MeOH induced a significantly higher CAP amplitude than that of 4-AP in both acute and chronic injuries, indicating that 4-AP-3-MeOH is more effective in restoring CAP regardless of stage of injury. A similar phenomenon has also been observed by Bei and his colleagues in an optic nerve regeneration model.⁵¹ It has been shown that both 4-AP and 4-AP-3-MeOH have no effect on CAP amplitude recorded from uninjured spinal cord axons,^{24,30} consistent with the notion that K_v plays an important pathological role in conduction loss in injured axons, but has no significant influence on action potential conduction in normal axons with intact myelin.

Although 4-AP has been shown before to provide motor behavioral benefits in live animals post-SCI,^{28,32} this is the first time that 4-AP-3-MeOH has been demonstrated with such ability. Perhaps equally important, this study is the first to demonstrate that the *in vivo* achievable safe dosage of 4-AP-3-MeOH (5 mg/kg) is at least 5 times higher than that of 4-AP (1 mg/kg) in the current rat SCI model. This is an obvious advantage, given that the magnitude of behavioral benefit offered by 4-AP is believed to be largely dictated by its achievable safe dosage *in vivo*.^{2,39} As such, the increased effectiveness of 4-AP-3-MeOH in providing behavioral benefits is likely attributed to, at least in part, its higher achievable dosages *in vivo*.

The toxicity of 4-AP in live animal has been reported in multiple previous observations.^{2,52–56} Because of the differences in application route, animal species, injury severities, motor behavioral assessments, direct comparison of dosage, effect, and toxicity may not be possible or appropriate. However, in the current study, 4-AP and 4-AP-3-MeOH were applied in parallel in the exact same type of injury and the same species, and the same behavioral tests were utilized, making direct comparison possible. Hence, by comparison, 4-AP-3-MeOH is safer and more effective than 4-AP in live animals, with an achievable safe dosage of 5 mg/kg. This is likely an important issue when advancing 4-AP-3-MeOH to clinical usage where toxicity is a key limiting factor for achieving meaningful benefits.

We caution that the comparison of potency, effectiveness, and side effects in the current study is mostly based on the data on *ex vivo* experiments and *in vivo* results are limited. A comprehensive comparisons of these parameters in live animals would require the knowledge of pharmacokinetic properties of both PCBs. Although the pharmacokinetics of 4-AP have been well studied,^{37,57,58} the similar properties of 4-AP-3-MeOH have yet to be evaluated. Once such information is available, a direct critical comparison of these two chemicals will then be appropriate in the evaluation of these drugs regarding the potential usage in clinical application.

It is interesting that both 4-AP-3-MeOH and 4-AP provided relief of post-SCI hypersensitivity. It is well known that post-SCI neuropathic pain is a significant symptom of SCI, which severely decreases quality of life beyond motor deficits.^{44,59–61} Therefore, treatments that can mitigate pain are highly warranted and can be independent of providing motor benefits. As such, the fact that both 4-AP-3-MeOH and 4-AP demonstrated the ability to reduce neuropathic pain-like behavior is of great importance. Despite their analgesic effect, the molecular mechanism of such an analgesic effect is unknown and remained to be explored.

One related phenomena associated with the *in vivo* behavioral benefit offered by the two PCBs examined in the current study is that although both 4-AP-3-MeOH and 4-AP can induce significant relief of post-SCI hypersensitivity at 1 mg/kg, such a dosage is not

FIG. 6. Increased motor function after 5 mg/kg of 4-AP-3-MeOH treatment 2 weeks after contusive SCI. Either 4-AP or 4-AP-3-MeOH were applied through intraperitoneal injection at dosages of either 1 or 5 mg/kg before rotarod motor function assessments: time on rotarod (**A**) and revolutions per minute (RPM; **B**). Compared to SCI animals, a significant improvement in motor function was observed in SCI animals treated with 5 mg/kg of 4-AP-3-MeOH, which was observed in both time on rotarod ($*p < 0.05$; $N = 5$; **A**) and RPM ($*p < 0.05$; $N = 5$) (**B**). Animals treated with 5 mg/kg of 4-AP exhibited severe seizure activity and were not fit to complete the assessment (data not shown). Error bars represent SEM. 4-AP, 4-aminopyridine; 4-AP-3-MeOH, 4-aminopyridine-3-methanol; SCI, spinal cord injury; SEM, standard error of the mean.

FIG. 7. Mechanical allodynia and thermal hyperalgesia are alleviated in SCI animals after treatment with either 4-AP or 4-AP-3-MeOH. Two weeks post-SCI, animals were treated with either 4-AP or 4-AP-3-MeOH at concentrations of 1 or 5 mg/kg through intraperitoneal injection. (**A**) Animals were immediately assessed for mechanical allodynia where von Frey filaments were used to measure mechanical threshold sensitivity in grams (g) every hour for 6 h and again at 24 h. A significant increase in threshold was observed when compared to SCI only after treatment with 5 mg/kg of 4-AP-3-MeOH ($*p < 0.01-0.0001$), 1 mg/kg of 4-AP-3-MeOH ($^{\Phi}p < 0.001$), or 1 mg/kg of 4-AP ($^{\ddagger}p < 0.05-0.001$). In addition, 4-AP-3-MeOH at 5 mg/kg produced a significantly greater increase of mechanical threshold than that at 1 mg/kg ($*p < 0.01-0.001$). Further, at the same dosage of 1 mg/kg, 4-AP-3-MeOH elicited a significant greater increase of mechanical threshold when compared to 4-AP ($^{\#}p < 0.001$). $N = 5$ in all groups. (**B**) Animals were also tested for thermal hyperalgesia by assessing paw withdrawal frequency (%) with the acetone-evoked evaporation cooling test every hour for 6 h and again at 24 h. A significant reduction in the frequency of paw withdrawal was observed after treatment with 5 mg/kg of 4-AP-3-MeOH ($^{\&}p < 0.01-0.0001$), 1 mg/kg of 4-AP-3-MeOH ($^{\Phi}p < 0.001$), or 1 mg/kg of 4-AP ($^{\ddagger}p < 0.001$). In addition, 4-AP-3-MeOH at 5 mg/kg produced a significant greater decrease of paw withdrawal frequency than that at 1 mg/kg ($*p < 0.01-0.001$). Further, at the same dosage of 1 mg/kg, 4-AP-3-MeOH elicited a significantly greater reduction of paw withdrawal frequency when compared to 4-AP ($^{\#}p < 0.01-0.001$). $N = 5$ in all groups. Animals treated with 5 mg/kg exhibited severe seizure activity and were not fit to complete the assessment. Error bars represent SEM. 4-AP, 4-aminopyridine; 4-AP-3-MeOH, 4-aminopyridine-3-methanol; SCI, spinal cord injury; SEM, standard error of the mean.

sufficient to induce significant improvement in post-SCI motor behavior. This suggests that, at least in our study involving spinal cord contusion injury, both PCBs are more effective in restoring physiological sensory function than motor capabilities. The mechanism of such differential effect is not clear. Deciphering such mechanisms will possibly provide great opportunities to specifically target certain channels in certain subtype neurons to battle neuropathic pain.

In conclusion, based on pre-clinical animal experimentations, strong evidence suggests that 4-AP-3-MeOH is capable of restoring axonal conduction after myelin damage by blocking fast potassium channels, a well-established therapeutic target in neurotrauma and degenerative disease. Further, compared to bench marker 4-AP, 4-AP-3-MeOH displayed multiple advantages in restoring axonal conduction and mitigating motor and sensory dysfunctions, making it an attractive candidate to be developed for clinical application. This effort is particularly warranted because the unique strength of 4-AP-3-MeOH may not only complement the effectiveness, but also overcome the limitations of 4-AP in treating MS, an FDA-approved indication for 4-AP application. Further, 4-AP-3-MeOH may represent the new strategy that could be developed in the treatment of SCI, a devastating neurotrauma with no established therapy.

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Author Disclosure Statement

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