RESEARCH ARTICLE

Transdermal delivery of 4-aminopyridine accelerates motor functional recovery and improves nerve morphology following sciatic nerve crush injury in mice

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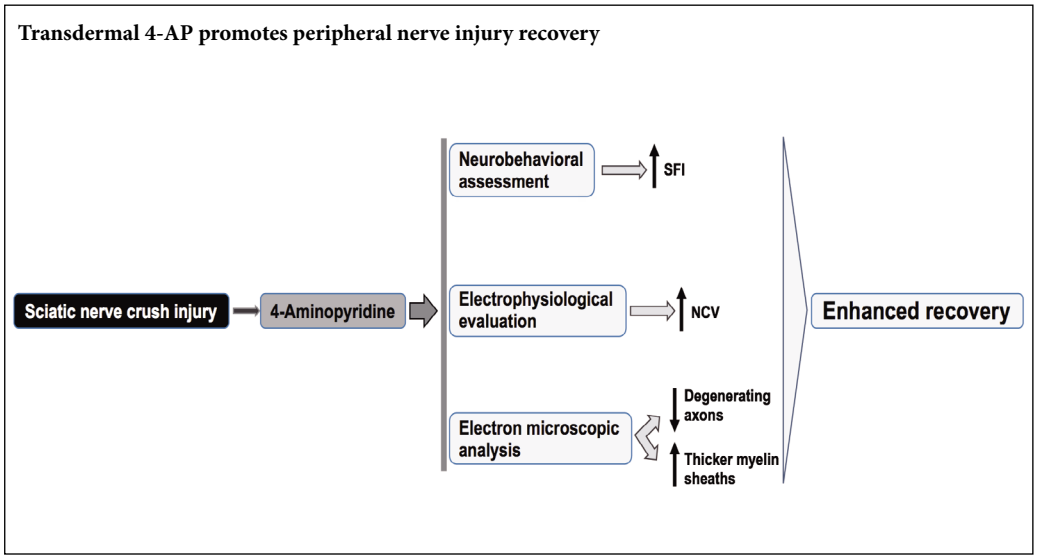
Graphical Abstract



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Abstract

Oral 4-aminopyridine (4-AP) is clinically used for symptomatic relief in multiple sclerosis and we recently demonstrated that systemic 4-AP had previously unknown clinically-relevant effects after traumatic peripheral nerve injury including the promotion of re-myelination, improvement of nerve conductivity, and acceleration of functional recovery. We hypothesized that, instead of oral or injection administration, transdermal 4-AP (TD-4-AP) could also improve functional recovery after traumatic peripheral nerve injury. Mice with surgical traumatic peripheral nerve injury received TD-4AP or vehicle alone and were examined for skin permeability, pharmacokinetics, functional, electrophysiological, and nerve morphological properties. 4-AP showed linear pharmacokinetics and the maximum plasma 4-AP concentrations were proportional to TD-4-AP dose. While a single dose of TD-4-AP administration demonstrated rapid transient improvement in motor function, chronic TD-4-AP treatment significantly improved motor function and nerve conduction and these effects were associated with fewer degenerating axons and thicker myelin sheaths than those from vehicle controls. These findings provide direct evidence for the potential transdermal applicability of 4-AP and demonstrate that 4-AP delivered through the skin can enhance *in-vivo* functional recovery and nerve conduction while decreasing axonal degeneration. The animal experiments were approved by the University Committee on Animal Research (UCAR) at the University of Rochester (UCAR-2009-019) on March 31, 2017.

Key Words: *4-aminopyridine; electron microscopy; functional recovery; nerve conduction velocity; peripheral nerve injury; pharmacokinetics; transdermal administration*

Chinese Library Classification No. *R452; R363; R641*

Introduction

Traumatic peripheral nerve injury (TPNI) represents a major public health problem that often leads to significant functional impairment and permanent disability (Robinson, 2000). The functional impairment with TPNI may result from multiple types of damage including the loss in axonal continuity, neuronal cell death, nerve demyelination, conduction defects, and muscle denervation (Robinson, 2000; Campbell, 2008; Lien et al., 2008). Although the promotion of re-myelination and enhanced functional recovery following TPNI could provide significant clinical benefits with early ambulation, there is no therapeutic strategy available for TPNI.

4-Aminopyridine (4-AP), a broad-spectrum potassium channel blocker and FDA-approved drug for the symptomatic treatment of multiple sclerosis (MS) (Egeberg et al., 2012; Jensen et al., 2014), has been shown to improve neuromuscular function in patients with diverse demyelinating disorders (Lundh et al., 1979; Hansebout et al., 1993; Sanders et al., 2000; Wirtz et al., 2009). Several studies have investigated the pharmacokinetic parameters of 4-AP and its derivatives in various experimental conditions (Uges et al., 1982; Davis et al., 1990; van Diemen et al., 1993; Pratt et al., 1995; Goodman and Stone, 2013). 4-AP is used either once-a-day or twice-a-day depending on the symptomatic response of the patient to 4-AP, and pharmacologic trials have shown that immediate release oral 4-AP has a mean time to maximum blood concentration of 1 hour, a mean half-life of 3.5 hours, and reversal of 4-AP effects gradually over 4–7 hours (Davis et al., 1990; van Diemen et al., 1993).

We have recently found that the recovery from acute TPNI caused by nerve crush injuries is enhanced by early treatment with 4-AP (Tseng et al., 2016). We demonstrated that both systemic and local 4-AP administration enhances global functional recovery of the affected limb, promotes remyelination of the nerve and improves the nerve conduction velocity in a mouse model of TPNI (Tseng et al., 2016; Noble et al., 2019). Unfortunately, despite these beneficial effects, the clinical utility of 4-AP to restore function after TPNI may be limited because of its narrow therapeutic window and the need for frequent oral dosing throughout the day (Goodman and Stone, 2013). With an aim to improve efficacy while reducing toxicity, several different formulations of oral 4-AP (Hayes et al., 2003; Goodman et al., 2010; Smith et al., 2010; Jensen et al., 2016) and pyridine-based derivatives (Smith et al., 2005) have been developed. However, although oral delivery is most commonly used, patients with TPNI are not universally free of gastrointestinal dysfunction due to trauma and they may also remain obtunded or under sedation with critical injuries, and the patient compliance decreases with increasing dosing frequency (Greenberg, 1984). Therefore, there is a need of alternative method of 4-AP delivery for its potential therapeutic benefits in TPNI.

Therapeutic benefits with transdermal delivery of multiple drugs are well documented (Prausnitz and Langer, 2008; Paudel et al., 2010), and locally or topically applied hormones, growth factors and immunosuppressants are reported to benefit post nerve-injury recovery (Galloway et al., 2000; Mohammadi et al., 2013; Mekaj et al., 2014). Although 4-AP is already in clinical use for demyelinating disorders (Lundh et al., 1979; Hansebout et al., 1993; Sanders et al., 2000; Wirtz et al., 2009), there is no report on the potential for transdermal delivery of 4-AP. Therefore, we asked whether 4-AP could be used as a transdermal agent and if so, what could be its effects on the functional and neuronal recovery after TPNI with once daily dosing. The first step in evaluating such effects would be to demonstrate that transdermal administration of 4-AP can promote functional recovery after TPNI as we have reported with 4-AP injection (Tseng et al., 2016).

This study was designed to explore and evaluate the applicability of transdermal delivery of 4-AP (TD-4-AP) both *in vitro* and *in vivo*, and also to investigate the *in vivo* effects of TD-4-AP on motor function, nerve conduction, myelination and axonal morphology following acute sciatic nerve crush injuries in a mouse model.

Materials and Methods

Animals

The experimental design and animal protocol was approved by the University Committee on Animal Research (UCAR) at the University of Rochester (UCAR-2009-019) on March 31, 2017 and the experiments were performed according to the guidelines of UCAR. A total of 54 ten-week-old female C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME, USA) weighing 20 to 25 g were used in this study and mice were housed at the animal facility according to UCAR guidelines.

In vitro skin permeability of 4-AP

Skin permeability testing for 4-AP (Sigma-Aldrich, St. Louis, MO, USA) was performed using unjacketed Franz diffusion cells (PermeGear, Hellertown, PA, USA) as per manufacturer's protocol with a receptor volume of 5 mL and orifice diameter of 11.28 mm. The hairs on the dorsum of the mouse were removed one day prior to experiment by a combination of shaving and application of Nair lotion. Immediately after euthanasia, the skin was excised, rinsed with PBS, placed over the Franz cell's receptor chamber containing 5 mL of PBS and equilibrated for 10 minutes. After equilibration, 4-AP (40 mg/mL) in 0.5 mL water or dimethyl sulfoxide (DMSO) was added in the donor chamber. Cumulative 4-AP diffusion across the skin was measured for 6 hours. The concentration of 4-AP in the collected samples was determined using UV-Vis with BioTek's Synergy™ Mx microplate reader (BioTek, Winooski, VT, USA) at 280 nm and 300 nm wavelengths with subtraction of skin background (DMSO or water) signals. Skin thickness was determined by a caliper. The cumulative amount of 4-AP traversing the skin was plotted as a function of time. The steady-state flux and lag time were calculated from the slope and x-intercept of linear extrapolation of the graph, respectively. Permeability coefficient (K_p) was calculated by dividing the flux by the initial amount 4-AP in donor compartment. The diffusion coefficient (D) was calculated by the equation: $D=H^2/(6 \times T_{lag})$, where H is

thickness of the skin and T_{lag} is the lag time.

Pharmacokinetics of transdermal 4-AP

To determine the pharmacokinetic parameters of 4-AP, a dose of 7.5 μL of 4-AP in DMSO (10 mg/mL or 20 mg/mL) was applied to a portion of lower back skin of anesthetized mice, and blood samples were collected at specified time points by cardiac puncture. 150 μL of acetonitrile was added to 50 μL of serum, vortexed for 1 minute, and then centrifuged at 10,000 r/min for 10 minutes at 4°C. The supernatant layer was filtered through a 0.2 μm syringe filter (Millex-FG, Millipore), and 10 μL of the solution was injected for liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) analysis. Liquid chromatography separation was performed isocratically at 800 μL/min on the Waters Atlantis HILIC column at 25°C. The mobile phase was 5 mM ammonium acetate in 10/90/0.2 (v/v/v) water/acetonitrile/formic acid. The mass spectrometry consisted of a Thermo Quantum Access Max Triple Quadrupole (GenTech Scientific, Arcade, NY, USA). The Ionspray voltage was set at 4500 V with the source temperature at 500°C. The sheath gas pressure and auxiliary gas pressure were set at 65 psi and 5 psi respectively. LC/MS/MS analysis was carried out with argon as the collision gas. Ion transitions were 95/78 for 4-AP and 110/93 for 3,4-diaminopyridine (3,4-DAP). 4-AP was quantified with a calibration curve (0.01–20 μM), and was qualitatively assessed by comparison of analytical response for 4-AP with that of the internal standard, 3,4 -DAP. The within-day and between-day precision was established by assaying quality control samples prepared at 0.01 μM (lower limit quantitation) and at 10 μM (higher limit quantitation) for three analyses with error within 15%.

Sciatic nerve crush injury and transdermal 4-AP application

Sciatic nerve crush injury was performed as previously described (Elfar et al., 2008). Briefly, after intraperitoneal ketamine (60 mg/kg)/xylazine (4 mg/kg) anesthesia, hair clipping and aseptic animal preparation, a lateral skin incision along the length of the femur was made, sciatic nerve was bluntly exposed through the iliotibial band and crushed proximal to the tibial and peroneal divisions using a smooth forceps with a metal calibration ring to standardize pressure for 30 seconds. The wound was closed and subcutaneous buprenorphine (0.05 mg/kg) was given for postoperative analgesia immediately after surgery and every 12 hours thereafter for the next 3 days. For the acute effect of TD-4-AP on post-injury day 1 (n = 6–7/group), SFI was measured before and within 2 two hours of a single dose of 4-AP (150 μg) application to a portion of lower back skin of mice. For the chronic effect of TD-4-AP (n = 8/group), a daily dose of 7.5 μL of 4-AP in DMSO (20 mg/mL) or DMSO only was applied to a portion of lower back skin of mice from post-injury day 1 to post-injury day 14.

Sciatic function index (SFI)

The effects of transdermal 4-AP were evaluated by SFI, a noninvasive means to determine the direct *in vivo* functional recovery after sciatic nerve injury (Inserra et al., 1998; Varejao et al., 2001; Elfar et al., 2008). The SFI is measured on a scale of 0 (normal) to 100 (complete loss of function). Briefly, mice were trained to walk freely along a 77 cm by 7 cm corridor lined with white paper and individual footprints were obtained by painting each foot (injured- black, uninjured- blue). Paw prints were measured for toe spread (distance from 1st toe to 5th toe) and paw length (length from third toe to bottom of the print). Three prints from the experimental (injured) and normal (uninjured) sides were measured, and SFI was calculated for each animal by averaging these measurements and using the following formula (Inserra et al., 1998): SFI = 118.9((ETS–NTS)/NTS))–51.2((EPL–NPL)/NPL))–7.5. Where E is the experimental paw, N is the normal paw, TS is toe spread, and PL is paw length.

Electrophysiological analysis

Nerve conduction studies with Nicolet Viasys Viking Select EMG EP system (Natus, Pleasanton, CA, USA) were performed by electrical stimulation of the nerve and recording compound muscle action potential (CMAP) from needle electrodes overlying the tibialis anterior muscle supplied by that nerve as previously described (Tseng et al., 2016). Briefly, subdermal stainless steel needle electrodes (6 V, 0.1 ms, 1 Hz, 1–10 mA) were inserted into the resting muscle of gluteal fold to obtain the first CMAP and then the stimulating electrodes were placed on popliteal fossa with a 10mm fixed distance from gluteal fold to obtain the second CMAP. Recording electrodes were placed along the tibialis anterior to measure CMAP. A reference electrode was placed in the back. The electrical current used to stimulate the nerve was ramped up until supramaximal levels were achieved. Nerve conduction velocity (NCV) was determined from the difference in latencies of two recorded CMAPs.

Transmission electron microscopy (TEM) and morphometric analysis

The crushed portion of sciatic nerves were excised and fixed for 24 hours in a combination fixative of 2.5% glutaraldehyde/4.0% paraformaldehyde buffered in 0.1 M sodium cacodylate and post-fixed 1.5 hours in buffered 2.0% osmium tetroxide, dehydrated in a graded series of ethanol up to 100%, transitioned into propylene oxide, then EPON/Araldite epoxy resin overnight. The next day, the nerves were embedded in fresh epoxy resin and polymerized for 2 days at 60°C. One micron sections were cut and stained with toluidine blue to assess the myelin before thin sectioning at 70 nm using an ultramicrotome and diamond knife. These sections were placed onto formvar/carbon slot grids, stained with uranyl acetate and lead citrate and digitally imaged using a Hitachi 7650 transmission electron microscope (Hitachi, Tokyo, Japan) with an attached Gatan Erlangshen digital camera (Gatan, Inc., Warrington, PA, USA) at 3000× magnification. Images were analyzed using ImageJ (NIH, Bethesda, MD, USA). Five images from each mouse were analyzed, containing a total of approximately 150–250 axons

per animal. Area and perimeter of inner and outer axon were measured. The inner and outer diameters were calculated from axon perimeter. Myelin thickness was calculated by the difference between outer and inner radius. G-ratio was calculated by the ratio of the inner axonal diameter to the outer diameter. Degenerating axons were counted as axons with significant malformed myelin sheath that invaded most of the neuron and showed detachment, and axons that had significant amount of internal debris (**Additional Figure 1**).

Statistical analysis

All data are expressed as mean ± standard error of the mean (SEM). One-compartmental pharmacokinetic analysis of the serum concentration-time data was performed using PKSolver (version 2.0, China Pharmaceutical University, Nanjing, China) to determine a complete pharmacokinetic profile. Data were analyzed using either two-tailed Student's *t*-test for paired data from the same experiment or unpaired data from different experiments or one-way analysis of variance followed by *post hoc T* test using Tukey correction for multiple comparisons. Nerve conduction and axon morphology data were analyzed by unpaired two-tailed Student's *t*-test, and reviewed with a qualified electro myographer certified for evaluation of these studies in humans. Values of $P < 0.05$ were considered to be statistically significant.

Results

4-AP penetrates *in vitro* skin with similar permeability coefficient in water or DMSO

Analysis of skin permeability is an important first step in the development of transdermal drug delivery. In this study, *in vitro* mouse skin permeation experiments with 4-AP were conducted using two different vehicles: water and DMSO. The cumulative amount of 4-AP permeation at each time point was plotted as a function of time to obtain skin permeability profiles of 4-AP (**Additional Figure 2**), and **Table 1** shows the parameters of skin permeation profiles. TD-4-AP was detectable in the receiving solution 1 hour after application and increased slowly over 6 hours demonstrating that 4-AP was able to penetrate the skin. The lag time, permeability coefficient, steady-state flux, and diffusion coefficient of the 4-AP in DMSO were lower than that in water, but there were no significant differences between two groups ($P > 0.05$).

Transdermal application of 4-AP increases serum 4-AP levels with linear pharmacokinetics

The relevant concentration of 4-AP in the serum following a single transdermal delivery was determined by a modified LC/MS/MS assay (Caggiano and Blight, 2013). This method utilized a low sample volume of 50 μL and simultaneously determined 4-AP and 3,4 DAP (as internal control) in the mouse serum with a chromatographic run time of 3.5 minutes (**Figure 1A–D**). **Figure 1A** and **B** shows the chromatograms of 4-AP and 3,4-DAP in serum samples from vehicle (DMSO)-treated mice, where there was no chromatogram peak for 4-AP. In contrast, serum samples from 4-AP treated mice exhibited a distinct chromatogram peak for 4-AP with a retention time of 2.84 minutes (**Figure 1C**) in addition to the internal control 3,4-DAP peak with a retention time of 2.6 minutes (**Figure 1D**). Ion transitions were 95/78 for 4-AP and 110/93 for 3,4-DAP. **Figure 1E** shows the serum 4-AP concentration versus time profile of the two dosages (75 or 150 μg) from 2–3 independent experiments. Selected pharmacokinetic parameters after transdermal 4-AP administration are shown in **Table 2** and it is evident that 4-AP has a linear kinetics.

TD-4-AP treatment promotes *in vivo* functional recovery after sciatic nerve injury

Figure 2 shows the functional evaluation of nerve injury and recovery as SFI at different experimental conditions. Acute application of vehicle DMSO alone (**Figure 2A**) did not affect SFI (–92.4 ± 1.66 to –90.6 ± 1.64, $P = 0.53$, $n = 7$), but a single dose of TD-4-AP (150 μg) significantly improved SFI within 2 hours of administration from –103 ± 1.63 to –95.1 ± 2.88 (**Figure 2A**, $P < 0.05$, $n = 6$). Furthermore, once-daily TD- 4-AP (150 μg) significantly accelerated the functional recovery from crush injury with respect to SFI as compared with DMSO alone (**Figure 2B**, $n = 8$/group) at post-injury day 3 (–56.5 ± 19.4 *vs.* –86.1 ± 8.0), day 5 (–65.1 ± 21.8 *vs.* –84.7 ± 6.9), and day 8 (–51.3 ± 18.7 *vs.* –70.9 ± 7.0). The SFI on day 14 was identical in both groups. These results demonstrate that, in addition to transient acute effect on motor function, daily TD-4-AP is effective in enhancing the functional recovery of sciatic nerve crush injury.

TD-4-AP treatment improves of nerve conduction after sciatic nerve injury

Electrophysiological evaluation was undertaken by stimulating the sciatic nerve and measuring the parameters from the tibialis anterior muscle as described in the Methods section. Treatment with TD-4-AP for 2 weeks caused a significant (~150%) improvement in the NCV compared to the DMSO group (**Figure 3A**; NCV 28.8 ± 5.2 m/s *vs.* 19.4 ± 3.6 m/s, $P < 0.05$, $n = 5$/group). This benefit was not dependent on the presence of 4-AP, as measurements were carried out at post-injury day 21 (7 days after the last dose of 4-AP). In contrast, we ob-

Table 1 *In vitro* skin permeability parameters of 4-aminopyridine (40 mg/mL) in water and dimethyl sulfoxide

Donor fluid	Skin thickness (mm)	Lag time (hour)	K_p (mm/h)	Flux (mg/mm^2 per hour)	Diffusion coefficient (mm^2/h)
Water	0.74±0.06	0.71±0.05	0.014±0.002	0.56±0.07	0.13±0.01
DMSO	0.64±0.03	0.56±0.06	0.011±0.001	0.43±0.01	0.12±0.004

Data presented as the mean ± SEM from three experiments. DMSO: Dimethyl sulfoxide; K_p: permeability coefficient; Flux: steady-state flux.

Table 2 Selected pharmacokinetic parameters after transdermal 4-AP administration

4-AP dose (μg)	T_{max} (minute)	C_{max} (μM)	AUC (μM•min)	MRT (minute)
75	53.68±5.45	5.32±1.10	760.00±81.99	107.42±10.91
150	55.76±2.96	7.97±0.39	1205.74±5.41	111.59±5.93

Data presented as the mean ± SEM from 2–3 independent experiments. 4-AP: 4-Aminopyridine; AUC: the area under the serum 4-AP concentration time curve; C_{max}: the maximum concentration; MRT: mean residence time; T_{max}: the time to maximum blood concentration.

served no difference between TD-4-AP and DMSO groups in CMAP area (**Figure 3B**: TD-4-AP *vs.* DMSO; proximal, 18.3 ± 3.34 mV·ms *vs.* 19.2 ± 2.59 mV·ms, $P = 0.84$, $n = 5$/group; distal, 17.8 ± 3.76 mV·ms *vs.* 19.49 ± 1.87 mV·ms, $P = 0.69$, $n = 5$/group) and CMAP amplitude (**Figure 3C**: TD-4-AP *vs.* DMSO; proximal, 14.2 ± 2.86 mV *vs.* 16.1 ± 2.81 mV, $P = 0.65$, $n = 5$/group; distal, 13.7 ± 3.18 mV *vs.* 17.1 ± 2.37 mV, $P = 0.41$, $n = 5$/group).

TD-4-AP treatment reduces axon degeneration and improves myelination after sciatic nerve injury

At post-injury day 21 (7 days after the last TD-4-AP), TEM representative images demonstrated an increased representation of well-preserved axons in the TD-4-AP-treated group (**Figure 4B**) as compared to the vehicle (DMSO) group (**Figure 4A**). While the total axon numbers were comparable between two groups at post-injury day 21 (**Figure 4C**), there were significantly fewer degenerating axons (**Figure 4D**) in the TD-4-AP group (49.0 ± 4.3) compared to the DMSO group (79.3 ± 11.8). Moreover, while the G-ratio was similar between the TD-4-AP and DMSO groups (**Figure 4E**), there was a significantly greater population of axons with larger myelin thickness (> 0.4 to < 0.8 μm) in 4-AP group (79.0 ± 5.3%) compared to DMSO group (67.7 ± 2.2%). There also was significantly smaller population of axons with thinner myelin sheaths (≤ 0.4 μm) in 4-AP group (13.9 ± 3.6%) compared to DMSO group (27.1 ± 4.5%) (**Figure 4F**).

Discussion

This study was undertaken to determine the applicability of transdermal 4-AP delivery, its pharmacokinetic characteristics, and the effects on the recovery of neuromuscular function following an acute sciatic nerve crush injury in mice. Here we report for the first time that 4-AP can be used as a transdermal therapeutic agent that promotes durable motor functional recovery of the limb with better preservation of the axonal myelin sheath thickness and improved nerve conduction in acutely injured sciatic nerve.

Although the pharmacokinetics and therapeutics of systemic 4-AP have been well characterized in various experimental and clinical studies (Davis et al., 1990; Pratt et al., 1995; Grijalva et al., 2003; Blight and Henney, 2009; Gobel et al., 2013; Goodman and Stone, 2013; Sindhurakar et al., 2017), this study was the first to explore the applicability and efficacy of transdermal 4-AP in a mouse model of sciatic nerve injury. First, to determine the skin permeability of the drug, we applied 4-AP through mouse skin using Franz diffusion cells. DMSO is a potent penetration enhancer (Williams and Barry, 2004), and nonetheless, 4-AP showed similar permeability coefficient in water or DMSO. Importantly, the permeability coefficient of 4-AP in water (0.014 mm/h) was close to the permeability coefficient of progesterone (0.015 mm/h), which is clinically used in transdermal patches (US EPA, 1992). Second, to evaluate the pharmacokinetic and therapeutic parameters of transdermal 4-AP, we applied 4-AP on the dorsum of mice and determined the serum concentration of 4-AP following transdermal application. After single transdermal application, we were able to identify serum 4-AP with a distinct chromatogram peak in addition to its internal control. Consistent with published reports (Uges et al., 1982; Davis et al., 1990; Blight and Henney, 2009), serum 4-AP levels slowly reached to its maximum concentration in 1 hour. The AUC and Cmax were directly proportional to the dose, demonstrating linear kinetics for 4-AP. The average time 4-AP stayed in the body after both doses of TD-4-AP was about 110 minutes. Taken together, these findings provide direct and conceivable evidence that 4-AP could be used as a transdermal therapeutic agent.

TPNIs occur along a spectrum from injuries in which some axonal continuity is maintained and injuries involving complete nerve transection. In those injuries in which continuity is maintained, as is the case for traumatic crush or compression injuries, we previously showed that treatment with 4-AP during the acute/sub-acute period post-injury (delivered by intraperitoneal injection or localized sustained release delivery) enhances functional recovery and promotes re-myelination in the standard animal model of such injuries (Tseng et al., 2016). In order to optimize the applicability of these discoveries, it is important to consider different drug delivery methods and that may be particularly applicable in individuals with traumatic injury. In this study, we demonstrate that once daily transdermal delivery of 4-AP can cause a transient as well as long lasting motor functional (SFI) improvement in mice with sciatic nerve crush injury compared to vehicle alone. Importantly, the benefits with chronic TD-4-AP are retained even after the treatment is stopped. It is possible that transdermal 4-AP may provide a more favorable pharmacokinetic profile compared to systemic 4-AP because of its several inherent advantages. Like other transdermal drugs (Prausnitz and Langer, 2008; Paudel et al., 2010), TD-4-AP could be particularly attractive because transdermal system is simple, noninvasive, inexpensive, and amenable to self-administration. In addition, it could be used in patients with gastrointestinal dysfunction due to trauma and also in obtunded and sedated patients with critical injuries.

Walking track analysis (SFI) is the gold standard for comprehensive evaluation of nerve recovery after sciatic nerve injury because proper walking requires coordinated functions involving sensory input, motor response, and cortical integration (Varejao et al., 2001). The significant improvements in motor function with TD-4-AP were accompanied by a significantly increased NCV but not by CMAP area or

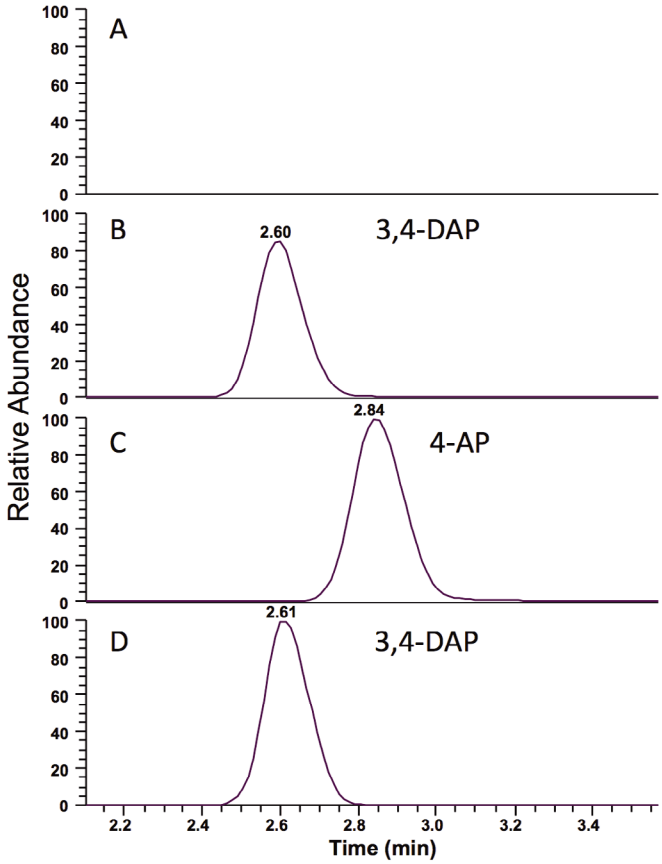

Figure 1 Chromatograms and serum concentrations of 4-AP from LC/MS/MS analysis.
Chromatograms of 4-AP (A) and 3,4-DAP (B) in serum samples from vehicle (DMSO)-treated mice. Chromatograms of 4-AP (C) and 3,4-DAP (D) in serum samples from TD-4-AP-treated mice. The time-course of serum 4-AP concentrations following transdermal administrations of 4-AP (E). n = 2–3 mice at each time point. 3,4-DAP: 3,4-Diaminopyridine; 4-AP: 4-aminopyridine; LC/MS/MS: liquid chromatography coupled with tandem mass spectrometry; TD-4-AP: transdermal delivery of 4-AP.

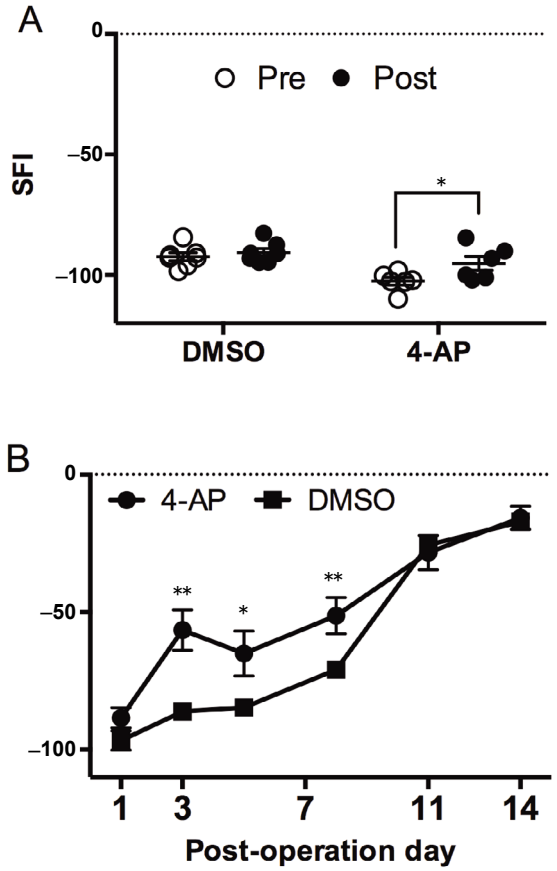

Figure 2 Effect of TD-4-AP and vehicle (DMSO) on the motor functional recovery after sciatic nerve crush injury.
(A) SFI following acute DMSO or acute TD-4-AP (150 µg) administrations, each symbol represents individual mouse (n = 6–7/group, $*P < 0.05$). (B) Time-course for the post-injury SFI recovery with daily TD-4-AP and DMSO treatments. Data presented as mean ± SEM, n = 8/group; $*P < 0.05$, $**P < 0.01$ *vs.* respective DMSO values (paired two-tailed Student's t-test for A, and one-way analysis of variance followed by *post hoc T* test using Tukey Correction for B). 4-AP: 4-Aminopyridine; DMSO: dimethyl sulfoxide; SFI: sciatic function index; TD-4-AP: transdermal delivery of 4-AP.

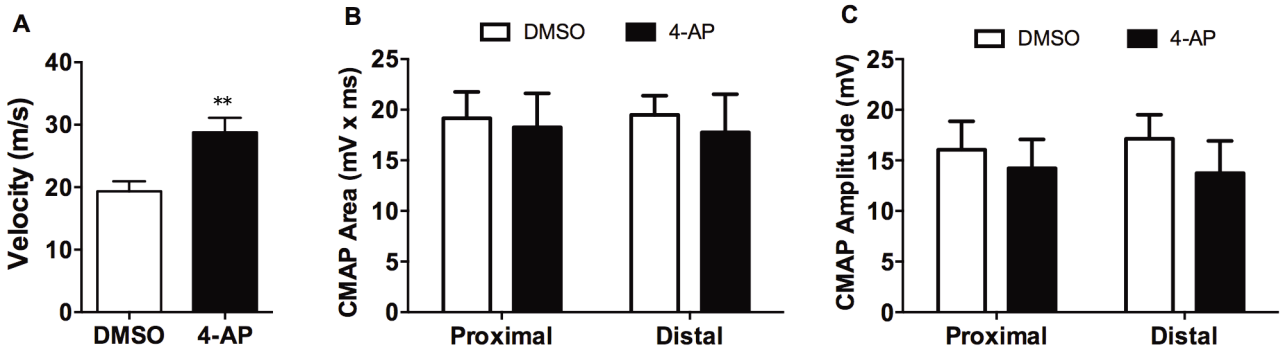

Figure 3 Effect of daily TD-4-AP treatment on electrophysiological function following sciatic nerve crush injury.
Bar graphs showing NCV (A), CMAP area (B) and CMAP amplitude (C) measured at post-injury day 21. Data presented as mean ± SEM, n = 5/group; $*P < 0.05$ *vs.* DMSO (unpaired two-tailed Student's t-test). 4-AP: 4-Aminopyridine; CMAP: compound muscle action potential; NCV: nerve conduction velocity; TD-4-AP: transdermal delivery of 4-AP.

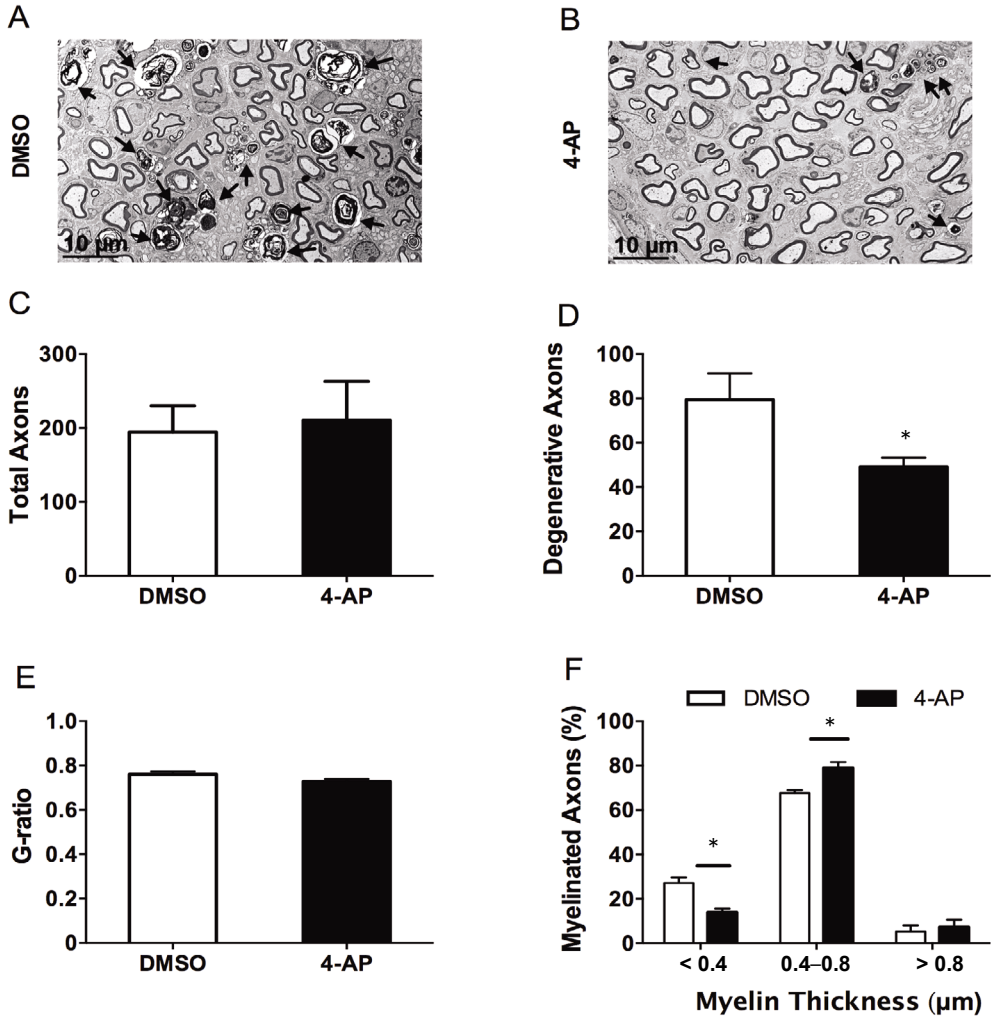

Figure 4 TEM analysis of transverse sections of sciatic nerves within the injury site at post-injury day 21 for the effect of TD-4-AP and vehicle (DMSO) treatments on axon degeneration and nerve myelination.
Representative TEM images of nerves from DMSO- (A) and 4-AP-treated (B) mice (original magnification, 3000×; scale bar, 10 µm; black arrows indicate degenerating axons). Quantification of total axon counts (C), degenerated axon counts (D), G-ratio measurements (E), and myelin thickness distributions (F). Data presented as mean ± SEM; DMSO group, n = 3; TD-4-AP group, n = 4; $*P < 0.5$ *vs.* DMSO (unpaired two-tailed Student's t-test). 4-AP: 4-Aminopyridine; DMSO: dimethyl sulfoxide; SFI: sciatic function index; TD-4-AP: transdermal delivery of 4-AP; TEM: transmission electron microscopy.

amplitude. While the amplitude of CMAP is determined by the number of muscle fibers innervated and is proportional to the number of motor axons regenerated (Navarro and Udina, 2009), NCV is mainly related to the number of myelinated fibers or changes in axon diameter (Waxman, 1980; Ikeda and Oka, 2012; Menorca et al., 2013). In fact the time course of functional recovery in our nerve injury model was too rapid to be explained by the axonal regeneration. Interestingly, quantitative TEM imaging analysis demonstrated a significantly decreased number of degenerative axons and an increased number of axons with thicker myelin sheaths in the TD-4-AP group compared to DMSO alone. The total number of axons and G-ratio were similar in both groups. The decrease in degenerating axons was particularly intriguing, and raises the question of whether 4-AP may have previously unrecognized neuroprotective effects when applied in the acute/sub-acute period after traumatic nerve injury. If the widely observed benefits of electrical stimulation in peripheral nerve injury models are due to stimulating nerve conduction (Al-Majed et al., 2000; Vivo et al., 2008; Singh et al., 2012), then the improved NCV with increased number of axons with thicker myelin in the TD-4-AP group may indicate a faster communication with muscle and the subsequent improvements in SFI. It is also possible that there may be enhanced clearance of the axonal debris, especially as there were no differences in the total number of neither axons nor the amplitudes.

In addition to multiple sclerosis (Egeberg et al., 2012; Jensen et al., 2014), 4-AP has been shown to improve neuromuscular function in patients with diverse demyelinating disorders including myasthenia gravis (Lundh et al., 1979), spinal cord injury (Hansebout et al., 1993), and Lambert-Eaton syndrome (Sanders et al., 2000; Wirtz et al., 2009). The neurological benefits of 4-AP are believed to result from increases in action potential duration, calcium influx, neurotransmitter release and synaptic transmission (Judge and Bever, 2006). Nerve trauma usually involves both mechanical (primary) and biochemical (secondary) events (Robinson, 2000; Navarro et al., 2007; Menorca et al., 2013), and 4-AP is reported to restore the conduction after physical or chemical damage (Yan et al., 2016; Modrak et al., 2019). The improved NCV with increased number of axons with thicker myelin in TD-4-AP group may indicate a faster communication with muscle and the subsequent improvements in SFI. Given the proven beneficial effects of 4-AP in neurodegenerative and demyelinating disorders and the faster functional recovery with TD-4-AP at intermediate time points compared to DMSO group in this study, it is tempting to speculate that faster recovery in mice with nerve crush injury could mean weeks/months faster recovery in humans.

Although our interesting findings with TD-4-AP are consistent with our earlier study with systemic 4-AP (Tseng et al., 2016) and provide substantial evidence for its usefulness in TPNIs, our study has some limitations. We did not investigate the concentration-dependent effects of TD-4-AP and used only a crush injuries and not the permanent denervation model. We also can only speculate as to the relationship between 4-AP dosage and actual *in vivo* blockade of potassium channels long thought to be the site of clinical efficacy for 4-AP in chronic disorders (Dunn and Blight, 2011). Finally, true sustained release formulations used for transdermal application contain materials that allow for a slow release of the drug to the skin to prolong the delivery profile over the course of hours or days. We did not evaluate external materials commonly used in designing clinically applicable means of slowing release over the course of an entire day.

In conclusion, this study was particularly designed to determine the feasibility of transdermal administration of 4-AP and its usefulness in TPNIs. Consistent with published reports (Uges et al., 1982; Davis et al., 1990; Blight and Henney, 2009), pharmacokinetic parameters in our experimental conditions showed linear kinetics for TD-4-AP with a Tmax at 60 minutes. Functional, electrophysiological and TEM findings demonstrated that TD-4-AP is effective in TPNI with significant positive effects on motor function and nerve recovery processes. These findings have significant clinical implications because this delivery method can be used to provide a sustained circulating blood level of drug with enhanced patient compliance and, importantly for drugs with shorter half-lives, without the need for multiple daily oral dosing or injections (Prausnitz and Langer, 2008; Paudel et al., 2010). TD-4-AP could be a promising alternative to systemic 4-AP, and this study thus provide the rationale for further investigations with suitable transdermal slow-release formulations in the setting of peripheral neurotrauma where no medical treatment is currently available.

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Additional files:

Additional file 1: *Open peer review reports 1 and 2.*

Additional Figure 1: *Representative transmission electron microscopy analysis of transverse sections of sciatic nerves within the injury site for axon degeneration and nerve myelination.*

Additional Figure 2: *Time-course for in vitro release studies of 4-aminopyridine (4-AP) from water and dimethyl sulfoxide (DMSO).*

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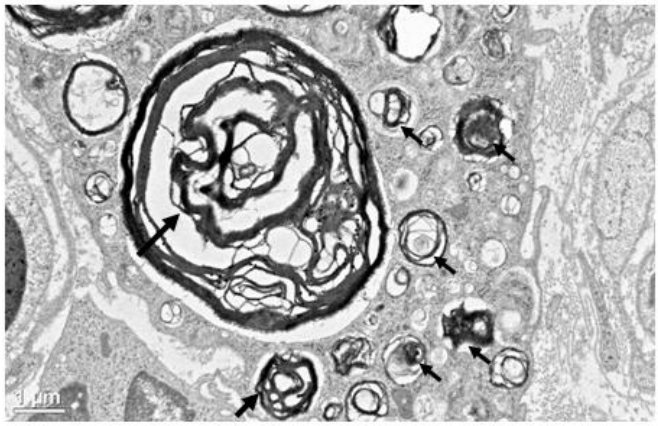
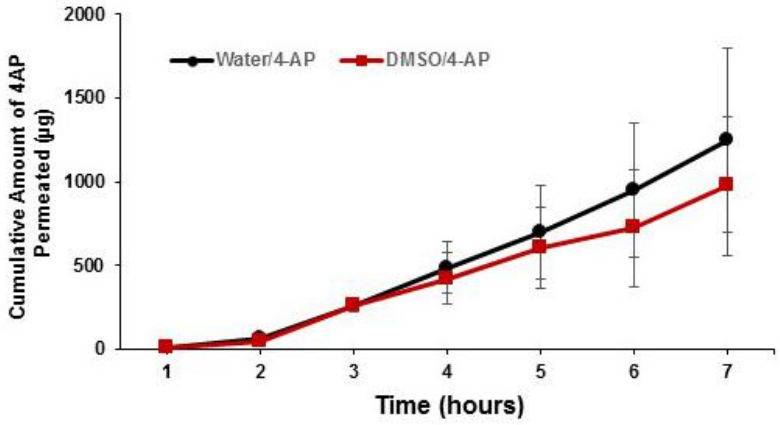


Additional Figure 1 Representative transmission electron microscopy analysis of transverse sections of sciatic nerves within the injury site for axon degeneration and nerve myelination.

Degenerating axons (arrows) were counted as axons with significant malformed myelin sheath that invaded most of the neuron and showed detachment, and axons that had significant amount of internal debris.



Additional Figure 2 Time-course for *in vitro* release studies of 4-aminopyridine (4-AP) from water and dimethyl sulfoxide (DMSO).

Data presented as mean ± SEM, n = 3/group.