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ORIGINAL ARTICLE

Acrolein involvement in sensory and behavioral hypersensitivity following spinal cord injury in the rat

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Abstract

Growing evidence suggests that oxidative stress, as associated with spinal cord injury (SCI), may play a critical role in both neuroinflammation and neuropathic pain conditions. The production of the endogenous aldehyde acrolein, following lipid peroxidation during the inflammatory response, may contribute to peripheral sensitization and hyperreflexia following SCI via the TRPA1-dependent mechanism. Here, we report that there are enhanced levels of acrolein and increased neuronal sensitivity to the aldehyde for at least 14 days after SCI. Concurrent with injury-induced increases in acrolein concentration is an increased expression of TRPA1 in the lumbar (L3–L6) sensory ganglia. As proof of the potential pronociceptive role for acrolein, intrathecal injections of acrolein revealed enhanced sensitivity to both tactile and thermal stimuli for up to 10 days, supporting the compound's pro-nociceptive functionality. Treatment of SCI animals with the acrolein scavenger hydralazine produced moderate improvement in tactile responses as well as robust changes in thermal sensitivity for up to 49 days. Taken together, these data suggest that acrolein directly modulates SCI-associated pain behavior, making it a novel therapeutic target for preclinical and clinical SCI as an analgesic.

Keywords: aldehyde, hydralazine, hyperreflexia, lipid peroxidation, proalgesic, TRPA1.

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Persistent neuropathic pain drastically impairs the quality of life for individuals suffering from spinal cord injury (SCI) (Hulsebosch *et al.* 2009). Despite years of research, this type of neuropathic pain remains refractory to treatment, and the exact source of which remains unknown. Though chronic SCI pain is felt throughout the body, patients commonly describe the pain as be located near or below the level of the injury (Siddall *et al.* 2003). In a rodent SCI model, changes in behavioral sensitivity arising below the level of the injury may be because of hyperexcitability of the spinal circuits because of interruption within the descending inhibitory tracts (Bruce *et al.* 2002; Lu *et al.* 2008; You *et al.* 2008), synaptic potentiation in dorsal horn neurons (Hains *et al.* 2003; Tan and Waxman 2012) or central sensitization as a result of moderate contusive injury and persistent hyperexcitability of nociceptors (Lu *et al.* 2008; Bedi *et al.* 2010).

The possibility that thoracic spinal cord injury could influence nociceptor sensitization was first demonstrated by elevated spontaneous activity in Ad and C fiber-associated neurons derived from the cervical, thoracic, and lumbar ganglia (Bedi *et al.* 2010). The incidence of spontaneous activity was greatest in lumbar dorsal root ganglia (DRG) neurons as compared to neurons from the cervical DRG; the increase in activity continued for up to 8 months (Bedi *et al.*

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Abbreviations used: DRG, dorsal root ganglia; IP, intraperitoneal; PID, post-injury day; PLC, phospholipase C; SCI, spinal cord injury; TRPA1, transient receptor potential ankyrin 1; TRPM8, transient receptor potential cation channel subfamily M member 8; TRPV1, transient receptor potential vanilloid subfamily, member 1.

2010). This type of prolonged hypersensitivity of nociceptor activity arising from sites of tissue or nerve injury has been described as a potential mechanism of a number of chronic pain conditions as it leads to long term changes in the central nervous system and contributes to amplification and persistence of pain via central sensitization (Devor 2009; Latremoliere and Woolf 2009). Although there are a myriad of maladaptive mechanisms including ionic imbalances, the release of pro-inflammatory cytokines and evidence of dysfunctional glia, there is little information regarding the possible role of lipid peroxidation products or accumulation of acrolein-protein adducts.

Acrolein is an aldehyde produced by lipid peroxidation products (Esterbauer *et al.* 1991; Hamann and Shi 2009; Shi *et al.* 2011a) and a agonist of the electrophile-sensitive transient receptor potential ankyrin 1 receptor (TRPA1), known to be present on a subpopulation of small unmyelinated both peptidergic and non-peptidergic nociceptors in the DRG (Bautista *et al.* 2006; Barabas *et al*. 2012). With its long half-life, acrolein is a potent endogenous toxin, known to lead to oxygen radical formation, perpetuate oxidative stress, and has been implicated in many neuropathological diseases (Hamann and Shi 2009; Shi *et al.* 2011a). The presence of acrolein may also influence thermal, mechanical, and inflammatory pain modalities (Bautista *et al.* 2006; del Camino *et al.* 2010; Vilceanu and Stucky 2010). Noted increases in the level of acrolein are known to exist following spinal cord injury and may serve to activate TRPA1 following SCI (Luo *et al.* 2005).

Hydralazine, used clinically to treat severe hypertension, is known to react with acrolein and prevent formation of carbonyl-retaining protein adducts in treated murine hepatocytes (Burcham and Pyke 2006). Hydralazine treatment also mitigates some of the cell death associated with acrolein-induced and compression-induced spinal cord injury (Hamann *et al.* 2008a,b). Though the degree to which acrolein contributes to SCI neuropathic pain behavior is unknown, hydralazine may serve as a therapeutic strategy for pain control provided it reduces the accumulation of aldehydic products of lipid peroxidation (Burcham *et al.* 2002; Liu-Snyder *et al.* 2006; Hamann and Shi 2009). Here, we test the hypothesis that acrolein can increase sensitization of DRG neurons derived from SCI animals, and that sequestration of SCI-induced acrolein using hydralazine reduces behavioral attributes of neuropathic pain behavior in the rodent.

Experimental procedures

Experimental animals and surgery

This study included data from Male Sprague–Dawley rats weighing 210–230 g. Rats were obtained from Harlan Laboratory (Indianapolis, IN, USA) and housed and handled in compliance with the Purdue University Animal Care and Use Committee guidelines and ARRIVE guidelines. They were kept at least 1 week before surgery for acclimation. Before surgery, rats were anesthetized with a ketamine (80 mg/kg) and xylazine (10 mg/kg) mixture by intraperitoneal (IP) injection. The spinous process and the vertebral lamina were removed to expose the dorsal surface of spinal cord at the T-10 spinal level. Following vertebral stabilization, the spinal cord was injured with a weight drop impactor (New York University impactor) using a 10-gram weight dropped from 25 mm onto the intact dura matter. A sham operation was performed using only a laminectomy of the T-10 vertebra without a spinal cord contusion. After surgery, the animals were allowed to recover on a heating pad. Post-surgical care of SCI rats included daily manual bladder expression until the return of reflexive control of bladder function was observed and 3-mL saline was administration via subcutaneous injection to prevent from dehydration.

Hydralazine application

The hydralazine hydrochloride (Sigma, St. Louis, MO, USA) solution was dissolved in phosphate buffered saline which was applied intraperitoneal at a final doses of 5 mg/kg. Two treatment regiments were used in SCI rats. In one of them, the hydralazine was applied daily for 2 weeks immediately following injury, while in the other daily application for 5 weeks was initiated 2-week post-SCI. In a separate experiment of assessing the effectiveness of suppressing acrolein, hydralazine was administrated twice, immediately following SCI, and again at 24-h post-SCI. The animal was killed 2 h following the second treatment and the acrolein level was determined through immunoblotting.

Behavioral quantification of nociception

Mechanical hyperreflexia

The foot withdrawal threshold to mechanical stimuli was used as an indicator of mechanical hyperreflexia. The SCI rats were placed on top of a metal mesh floor and covered by a transparent plastic box. The animals were left alone in this setting for at least 10 min to allow for acclimation before testing. For mechanical stimulation, a series of calibrated von Frey filaments (range: 0.4, 0.6, 1.0, 2.0, 4.0, 6.0, 8.0, and 15.0 grams, Stoelting, Wood Dale, IL, USA) were applied perpendicular to the plantar surface of the hind limb, with sufficient bending force, for 3–5 s. Stimuli were applied at a frequency of 1 per minute. A brisk hind limb withdrawal with or without licking and biting was considered to be a positive response. In the event of paw withdrawal (positive response) in response to one level of stimulus, lower grade stimulation was followed until no positive response can be elicited. Then, the filament of the next greater stimulus was applied again to confirm the positive response which will then be used as the mechanical thresholds.

Cold hyperreflexia

Cold sensitivity was assessed using the 100% acetone-evoked evaporative cooling test. In a setting similar to that in the assessment of mechanical hyperreflexia, 0.05 mL acetone was applied from a distance of 2 mm from the plantar surface of the hind paw. The acetone was applied five times to each paw at intervals of 5 min. The paw withdrawal or hind paw licking response to the application of acetone was interpreted as a sign of cold hyperreflexia.

During the experimental process, the influence of one test on the following one was minimized by performing the test that was least

stressful to the animal first. Therefore, order of the behavioral tests was von Frey filament assay and then acetone application assay. The sequence of the tests was kept the same throughout the experimental period for all animals. Furthermore, the animals were allowed to rest for at least 20 min between different behavioral tests.

Acrolein microinjection

Animals (210–230 g) were anesthetized with a cocktail of Xylazine (10 mg/kg) and Ketamine (80 mg/kg) intraperitoneally. A dorsal laminectomy was performed at the 10th thoracic vertebra exposing the spinal cord for injection. In addition, taking care to avoid contact with the cord, the dural sheath was lanced with a needle point to facilitate micropipette insertion. Micropipettes were pulled using a programmable puller (Model P-80, Sutter Instruments, Novato, CA, USA). The tip of the micropipettes (outer diameter: ~ 50 μm) were then beveled at a 45 degree angle to minimize the occurrence of obstructions and reduce mechanical insult to tissue. The pipettes were loaded manually with sterile saline taking care to ensure no bubbles were present except for the fluid level indicator. Acrolein was then loaded using a three-way valve and a syringe under negative pressure. Injections were delivered via the PMI-100 pressure micro-injector (Dagan Corp., Minneapolis, MN, USA). Individual injections of sterile saline and acrolein (volume: 1.6 μL) were made on opposing sides of the spinal cord 0.6 mm lateral to midline and 1.2 mm ventral to the cord surface. Following injection, the micropipette was backed out of the injection site and tested to ensure patency. The surgical site was flushed with sterile saline and muscle then dermal layers were sutured sequentially with interrupted sutures. The entire operation of microinjection was conducted under dim light to minimize light exposure to acrolein.

Isolation of spinal cord

The animals were anesthetized with an I.P injection of a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg). When they were deeply anesthetized, rats were perfused transcardially with cold, oxygenated Kreb's solution (124 mmol/L NaCl, 2 mmol/L KCl, 1.24 mmol/L KH2PO4, 26 mmol/L NaHCO3, and 10 mmol/L ascorbic acid, 1.3 mmol/L MgSO4, 1.2 mmol/L CaCl2, and 10 mmol/L glucose). The whole vertebral column was then rapidly excised, and the spinal cord was removed to cut into 1 cm segments including the injury site for the experiments such as the determination of tissue acrolein levels describe below.

Protein immunoblotting

The spinal cord segments including injury site (1 cm long) was incubated with 1% Triton solution with the corresponding amount of Protease Inhibitor Cocktails (Sigma-Aldrich, Product #: P8340) and then homogenized with a glass homogenizer (Kontes Glass Co., Vineland, NJ, USA). The solution was then incubated on ice for at least 1 h before being centrifuged at 13 500 *g* for approximately 30 min at 4°C. Samples were stored at −80°C and kept for no more than 2 weeks before the experiment. An additional round of centrifugation at the same parameters was performed after removal from storage.

Bicinchoninic acid protein assay was performed to ensure equal loading concentrations for all samples. Samples were transferred to a nitrocellulose membrane using a Bio-Dot SF Microfiltration Apparatus (Bio-Rad, Hercules, CA, USA). The membrane was blocked for 1 h in blocking buffer (0.2% casein and 0.1% Tween 20 in Phosphate buffered saline), and then transferred to primary antibody solution for acrolein (monoclonal mouse anti-acrolein antibody from ABCAM, Cambridge, MA, USA) for 18 h at 4°C. The antibody was diluted to a concentration of 1 : 1000 in blocking buffer with 2% goat serum and 0.025% sodium azide. The membrane was washed in blocking buffer prior to being transferred to an alkaline phosphatase (conjugated to goat anti-mouse IgG) solution diluted to 1 : 10 000 for 1 h (VECTASTAIN ABC-AmP Kit; Vector Laboratories Inc., Burlingame, CA, USA). Final washes of the membrane were performed with blocking buffer and followed by 0.1% Tween 20 in Tris-buffered saline. The membrane was then exposed to Bio-Rad Immuno-Star Substrate or ABC-AMP kit substrate, and visualized via chemilluminescence. Density of bands was evaluated using Image J processing program (NIH) expressed as arbitrary unit.

RNA isolation and cDNA synthesis

Total RNA was isolated from the L1-L6 DRGs of naïve and SCI rats 7 days after surgery using Trizol reagent (Sigma-Aldrich, St. Louis, MO, USA). RNA isolation was followed by chloroform extraction and isopropanol precipitation. RNA concentration was then measured with a spectrophotometer at the optical density of 260. One microgram of total RNA from each sample was reverse transcribed using random primers and iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. Resulting cDNA products were diluted with RNAse-free water for each sample.

Real-time quantitative PCR

Primers were designed to recognize the TrpA1 receptor gene using previous sequences from (Nozawa *et al.* 2009) 5′-TCCTATACTGGAAGCAGCGA-3′, and 5′-CTCCTGATTGCCATCGACT-3′; 18S was used as an endogenous control gene with primers designed against the following sequences: 5′-CGGCTACCACATCCAAGGAA-3′ and 5′-GCTGGAATTACCGCGGCT-3′. Real-time PCR was performed by amplifying cDNA from each sample with the SYBR Green fluorescent system on a 7300 Read Time PCR System (Applied Biosystems). TrpA1 gene expression was normalized by the expression of 18S ribosomal RNA expression. Relative quantification was calculated as $X = 2 - \Delta\Delta Ct$, where $\Delta\Delta Ct = \Delta E - \Delta Ct$ and $\Delta E = Ct\ exp - Ct18s$, $\Delta Ct = Ct\ control - Ct\ 18\ s$ (Livak and Schmittgen 2001). Data were then normalized to the average of the control group.

Preparation of acutely dissociated dorsal root ganglion neurons

The L3–L6 DRGs were acutely dissociated using methods described by Ma and LaMotte (Ma and LaMotte 2005). The L3–L6 DRGs were removed from naive animals and SCI animals Post-injury day (PID) 21 days. The DRGs were treated with collagenase A and collagenase D in Hank's Balanced Salt Solution (HBSS) for 20 min (1 mg/mL; Roche Applied Science, Indianapolis, IN, USA), followed by treatment with papain (30 U/mL, Worthington Biochemical, Lakewood, NJ, USA) in HBSS containing 0.5 mM EDTA and cysteine at 35°C. The cells were then dissociated by mechanical trituration in culture media containing 1 mg/mL bovine serum albumin and trypsin inhibitor (Worthington Biochemical, Lakewood, NJ, USA). The culture media consisted of Ham's F-12

mixture and Dulbecco's Modified Eagle's Medium, supplemented with 10% fetal bovine serum, penicillin and streptomycin (100 μg/mL and 100 U/mL) and N2 (Life Technologies). The cells were then plated on coverslips coated with poly-L lysine and laminin (BD Biosciences, San Jose, CA, USA) and incubated for 2–3 h before additional culture media was added to the wells. The cells were then allowed to sit undisturbed for 12–15 h to adhere at 37°C with 5% CO_2.

Intracellular Ca^{2+} imaging

The dissociated DRG cells were loaded with Fura-2 AM (3 μM, Molecular Probes/Invitrogen Corporation, Carlsbad, CA, USA) for 25 min at 20°C in a balanced sterile salt solution [NaCl (140 mM), Hepes (10 mM), CaCl2 (2 mM), MgCl2 (1 mM), glucose (10 mM), KCl (5 mM)]. The cells were rinsed with the balanced sterile salt solution and mounted onto a chamber that was placed onto the inverted microscope. Intracellular calcium was measured by digital video microfluorometry with an intensified CCD camera coupled to a microscope and MetaFluor software (Molecular Devices Corporation, Downington, PA, USA). Cells were illuminated with a 150 W xenon arc lamp, and the excitation wavelengths of the Fura-2 (340/380 nm) were selected by a filter changer. Sterile solution was applied to cells prior to acrolein application; any cells that responded to buffer alone were not used in neuronal responsive counts. Acrolein (250 μM) was applied directly into the coverslip bathing solution. If no response was seen within 2 min, the acrolein was washed out. After acrolein application, capsaicin (3 μM) was added. Calcium imaging traces were analyzed by two independent analyzers and only responses that were in agreement between two individuals were used in the counts.

Electrophysiology

Sharp-electrode intracellular recordings were obtained 12–18 h after dissociation. Coverslips were transferred to a recording chamber that was mounted on the stage of an inverted microscope (Nikon Eclipse Ti, Nikon Instruments Inc., Melville, NY, USA). The chamber was perfused with a bath solution comprised NaCl 120 mM, KCl 3 mM, CaCl2 1 mM, MgCl2 1 mM, Hepes 10 mM, Glucose 10 mM, and adjusted to a pH of 7.4 and an osmolarity of 300 mosM. All recordings were obtained at 20°C. Intracellular recording electrodes were fabricated from borosilicate glass (World Precision Instruments, Sarasota, FL, USA) and pulled on a Flaming/Brown micropipette puller (P-98, Sutter Instruments, Novato, CA, USA). Electrodes were filled with 1.0 M KCl (impedance: 40–80 MΩ) and positioned by a micromanipulator (Newport Corporation, Irvine, CA, USA). −0.1 nA current injection was used to bridge-balance the electrode resistance. Prior to electrode impalement, the size of the soma to be recorded was classified according to its diameter as small (≤ 30 μm), medium (31–45 μm) and large (≥45 μm). Electrophysiological recordings were performed with continuous current-clamp in bridge mode using an AxoClamp-2B (Molecular Devices, Sunnyvale, CA, USA) amplifier, stored digitally via Digidata 1322A interface, and analyzed offline with pClamp 9 software (Axon Instruments, Union City, CA, USA). A neuron was accepted for study only when it exhibited a resting membrane potential more negative than −45 mV. For each isolated neuron studied, a continuous recording was obtained for one min without the delivery of any external stimulus. Action potentials were evoked by injecting current steps of 1 s duration through the intracellular recording electrode in increments of 0.1 nA, starting at 0.1 nA, until evoking one or more AP(s), or reaching 2 nA. The current threshold (CT, nA) was defined as the minimal current injection required to evoke a single AP. If spontaneous discharge persisted during this period, the neuron was classified as spontaneously active. Neuronal excitability of small and medium diameter dissociated DRG sensory neurons was measured by injecting 1 s current pulses into the soma every 30 s. Current was adjusted in order to elicit 3–4 action potentials per current injection under baseline conditions. Following three control current injections, acrolein (250 μM) was applied to the coverslip and current injections continued every 30 s. Neuronal excitability was measured as the number of action potentials elicited per current pulse before and after addition of acrolein.

Statistical analysis

ANOVA for repeated measures was used to determine the time course of SCI neuropathy as well as drug effects. One-way ANOVA was used to identify the source of significant interactions at each time point, followed by Tukey *post hoc* tests. The Student's *t*-test was used when comparing only two conditions. The differences in the incidence of neurons responding to acrolein were compared using the Yates-corrected chi-square test. All statistical analyses were performed using IBM-SPSS Statistics version 19.0 (SPSS inc., an IBM company, Chicago, IL, USA). $p < 0.05$ was considered statistically significant and the averages were expressed in mean ± SEM.

Results

Persistent elevation of acrolein protein adducts in rat spinal cord following trauma

A 1 cm segment of spinal cord tissue centered with the injury site and age matched sham-injured groups were collected to determine the level of acrolein adducts using a dot immunoblotting assay (Fig. 1). The acrolein levels of SCI rats were examined at the time-points 1 day PID and 2 weeks PID. The acrolein-lysine adduct level was significantly elevated at both 1 day (39.43 ± 2.46 a.u.) and 2 weeks (30.38 ± 2.71 a.u.) when values were compared between injured and sham-injury control animals (9.68 ± 4.67 a.u., $p < 0.001$).

Changes in TRPA1 mRNA expression level in the DRGs following the SCI

To better understand the potential influence of acrolein in the rodent SCI pain model, we studied changes in expression of TRPA1 present in the associated DRG using quantitative real-time PCR (Fig. 2). Seven days after SCI, TRPA1 mRNA expression levels were increased in DRGs ($n = 4$) by 2.03 ± 0.11 fold as compared to the sham-injured control group ($n = 4$).

Acrolein increases $[Ca^{2+}]_i$ in DRG cells after SCI

Because SCI increased the level of acrolein in the spinal cord and TRPA1 in DRG, we determined the degree to which

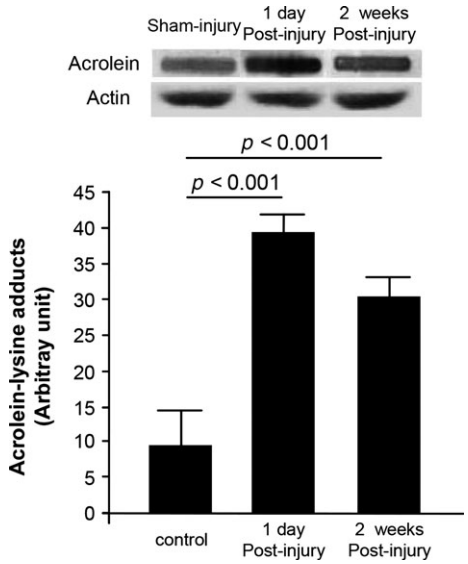

Fig. 1 Persistent elevation of acrolein adducts following thoracic spinal cord injury (SCI) in rats. SCI induces acrolein elevation in spinal cord tissue associated with contused tissue for at least 2 weeks. Dot immunoblotting assay demonstrated that SCI acrolein lysine adduct levels were elevated as early as 24 h after injury when compared with samples derived from sham injury animals at 1 day. These elevations were observed for at least 2 weeks after SCI. All data were expressed as mean ± SEM. $n = 6$ in all cases.

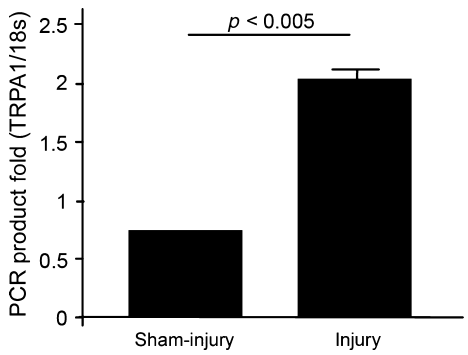

Fig. 2 RT-PCR relative quantification of TRPA1 mRNA in lumber (L3–L6) dorsal root ganglia (DRG) 7 days after spinal cord injury (SCI). Gene expression was normalized by the expression of 18 s and compared to the cycle threshold (Ct) value for 18 s of SCI tissue mRNA. The difference of gene expression is shown as the fold ratio. Relative transcript level was calculated as $X = 2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta E - \Delta Ct$ and $\Delta E = Ct$ exp–Ct 18 s, $\Delta Ct = Ct$ control–Ct 18 s. There is a twofold increase in TRPA1 mRNA present 7 days after SCI when compared to sham injury control group ($n = 4$). For the statistical analysis, paired *t*-test was used ($p < 0.005$).

exogenous acrolein increased intracellular Ca^{2+} concentration in acutely dissociated rat DRG neurons derived from injured or sham-injured control animals. We found that while 38% of sensory neurons responded to acrolein alone (39/103), approximately 14% (14/103) of the neurons from sham-injury rats also responded to capsaicin. Though the percentage of cells that responded to both acrolein and capsaicin did not change significantly in SCI rats (17% or 16/93), acrolein-responsive neurons did increase to 58% (54/93). This is significantly higher than what is observed in sham-injured rodents ($p < 0.005$, Chi-square with Yates correction). This data indicated that there was an increased number of sensory neurons that respond to acrolein following SCI, while the ratio of capsaicin responsive cells among acrolein responding cells was unaltered. In addition the number of sensory neurons that responded to capsaicin alone did not differ significantly between sham-injury and SCI animals (32/103 or 31% for sham-injury and 26/93 or 28% for SCI; $p > 0.05$).

Decreased current thresholds in DRG sensory neurons derived from SCI rodents

We then determined whether SCI affected the overall activity state of sensory neurons in rats following SCI. Electrophysiological current clamp recordings were compared for DRGs derived from both SCI and sham-injury control rats. Small diameter sensory neurons derived from SCI rats exhibited a significant decrease in the amount of current needed to elicit an action potential (CT) when compared with neurons from sham-injury animals (0.9 ± 0.1 nA for sham injury vs. 0.4 ± 0.1 nA for SCI, Figure S1A[1] and A[2], $p < 0.05$). Group data are shown in Figure S1b. Moreover, medium diameter sensory neurons derived from SCI rats also exhibited a significant decrease in the amount of current needed to elicit an action potential when compared with neurons from sham-injury animals (1.1 ± 0.1 nA for sham injury vs. 0.6 ± 0.1 nA for SCI, Figure S1C[1] and C[2], $p < 0.05$). Group data are shown in Figure S1d. There was no difference observed in CT in large diameter sensory neurons derived from sham injury and SCI rats. The mean CT for large DRG neurons was 2.10 ± 0.33 nA for SCI animals ($n = 15$) and 1.99 ± 0.17 nA for sham-injury animals ($n = 20$). This data indicate that SCI alone can alter the state of DRG small and medium diameter sensory neurons and increase their overall excitability.

Increased DRG neuronal excitability following a combination of acrolein and SCI

In addition to the increased number of neurons that responded to acrolein and exhibited a decreased CT following SCI, we were also interested in whether sensitivity to acrolein increased in single DRG sensory neurons. To determine the degree to which acrolein can induce an increase in sensory neuron excitability at single cell level, we examined neuronal response using sharp electrodes in current clamp mode. Acrolein did not produce spontaneous activity in any of the tested neurons. Following the combination of

repeated current pulses with administration of acrolein, we observed a significant increase in the excitability of some small to medium diameter sensory neurons when compared to baseline levels (current injection only) in both sham injury and SCI-derived sensory neurons (Fig. 3). Specifically, the average number of action potentials that can be elicited by minimal current injection in sensory neurons derived from sham injury animals is 1.9 ± 0.1 APs (n = 12) for control and 4.7 ± 1.0 APs following acrolein administration (n = 3) (Fig. 3b). In rats subjected to SCI, the number of action potentials present in sensory neurons following current injection is 2.3 ± 0.4 APs for control (n = 14) and 8.0 ± 0.9 APs after acrolein application (n = 5) (Fig. 3b). In both sham injury animals and SCI rats, the excitability of these neurons was significantly increased by acrolein when compared with controls (*$p < 0.05$ for both sham injury and SCI groups). In addition, the acrolein-mediated increase of excitability is greater in SCI versus sham injury rats ($p < 0.05$) (Fig. 3b). Thus, acrolein was interpreted as exciting subpopulations of nociceptive neurons; this same cellular excitation was found to be significantly enhanced in SCI rats.

Microinjection of acrolein into spinal cord produces nociceptive pain behavior

To examine the ability of acrolein alone to elicit nociceptive behavior in the absence of mechanical trauma, we injected a small amount of acrolein directly into the spinal cord dorsal horn of uninjured, healthy rats. Acrolein injections into the spinal cord produced significant tactile and thermal hyperreflexia on the side of the injection site, most likely because of the expression of TRPA1 channels in central terminals of primary afferents. Administration of saline to the contralateral side resulted in the absence or a reduced level of nociceptive pain behavior (Fig. 4).

Hydralazine lessens post-SCI neuropathic hyperreflexia

In order to determine whether scavenging acrolein could influence behavioral hyperreflexia following SCI, hydralazine was administered to SCI animals (i.p.) daily for a period of 2 weeks. Both injury-induced tactile and thermal hyperreflexia was evident by day 14 in untreated SCI animals. In a separate animal group, rats were subjected to SCI immediately followed by daily systemic hydralazine injections for 2 weeks. Such an intervention significantly diminished tactile (Fig. 5a) and thermal hyperreflexia (Fig. 5b) when compared with the SCI rodent group at days 14, 21, and 28 ($p < 0.05$). In addition, hydralazine treatment in the rodent SCI group significantly reduces acrolein-lysine adduct levels as early as 1 day after SCI when compared to the injury only group (Fig 5c, $p < 0.05$).

The seemingly beneficial effects of hydralazine administration on tactile and thermal hyperreflexia following SCI were not limited to the time period immediately after the traumatic injury. Delayed daily systemic hydralazine treatment began at day 14 and continued through day 49. This treatment paradigm significantly altered injury-induced tactile hyperreflexia from day 35 through the end of behavioral testing at day 49 (Fig. 6A; $p < 0.05$). Changes in injury-induced thermal hyperreflexia were robustly altered by hydralazine treatment within the first week of drug administration and continued through the end of behavioral testing (Fig. 6b; $p < 0.05$).

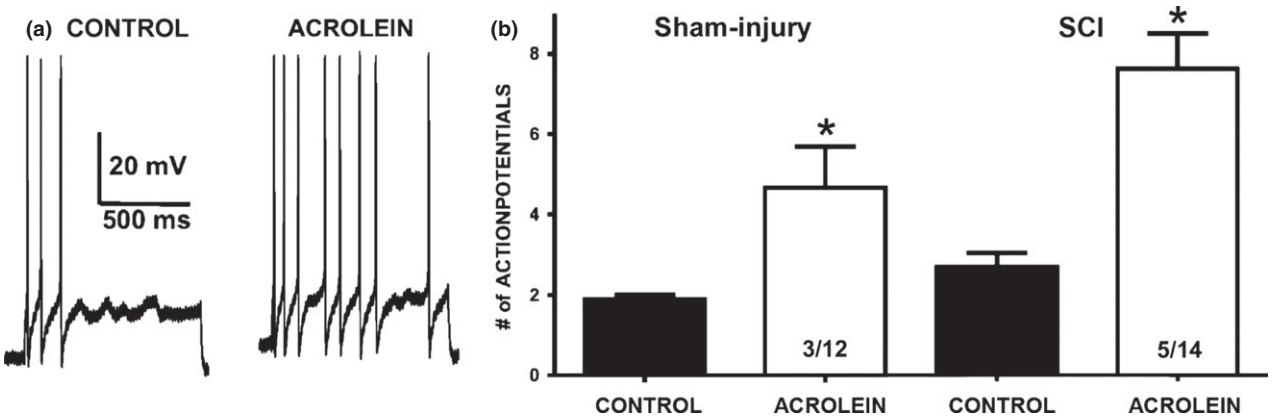

Fig. 3 Acrolein-induced excitability of nociceptive dorsal root ganglia (DRG) neurons is present by 14 days after spinal cord injury (SCI). Sharp-electrode intracellular recordings were obtained from DRG neurons 12–18 h after dissociation from either sham-injured or SCI rodents. Current clamp recordings were performed on small-to-medium diameter sensory neurons (>30 μm – >40 μm) derived from lumbar sensory ganglia (L3–L6). Firing of two to four action potentials (APs) were elicited by a 1 s depolarizing current injection (ranging from 0.1 to 2.0 nA depending on the cell) every 30 s. (a). Representative recordings demonstrating that application of acrolein (250 μM) increases the number of elicited action potentials in DRG sensory neurons derived from SCI rodent. (b) Group data showing that acrolein caused a significant increase in DRG action potential firing under both sham injury and SCI conditions (*$p < 0.05$ vs. control, ANOVA; n = 12 for sham injury controls, and n = 14 for SCI controls. In addition, the acrolein-mediated increase of excitability is greater in SCI versus sham injury rats (*$p < 0.05$, ANOVA).

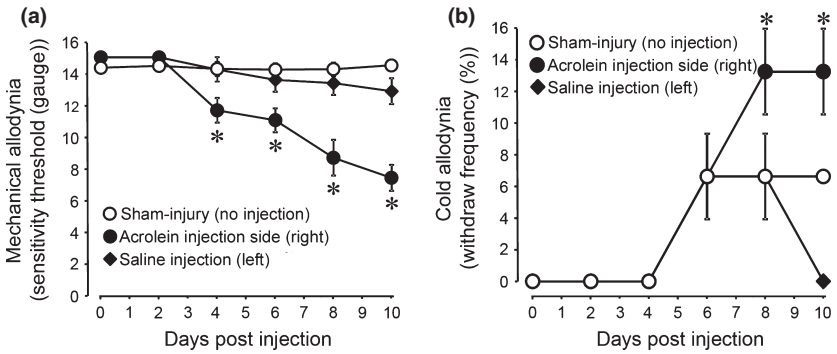

Fig. 4 Microinjection of acrolein directly into the thoracic spinal cord produces significant changes in behavioral response following hind paw stimulus. Behavioral changes associated with saline (left side) and acrolein (right side) injections into thoracic spinal cord were assayed across time using mechanical (a) and cold hyperreflexia (b) up to 10 days. Acrolein (40 nmol, 1.6 μL) was injected into the right side of dorsal aspect of spinal cord at T_{10} level and an equal volume of saline was injected into the left side of the cord. One-way ANOVA and Tukey's test were used for statistical analysis. (*$p < 0.05$ when compared to control group, $n = 4$ in all groups). All data were expressed as mean ± SEM.

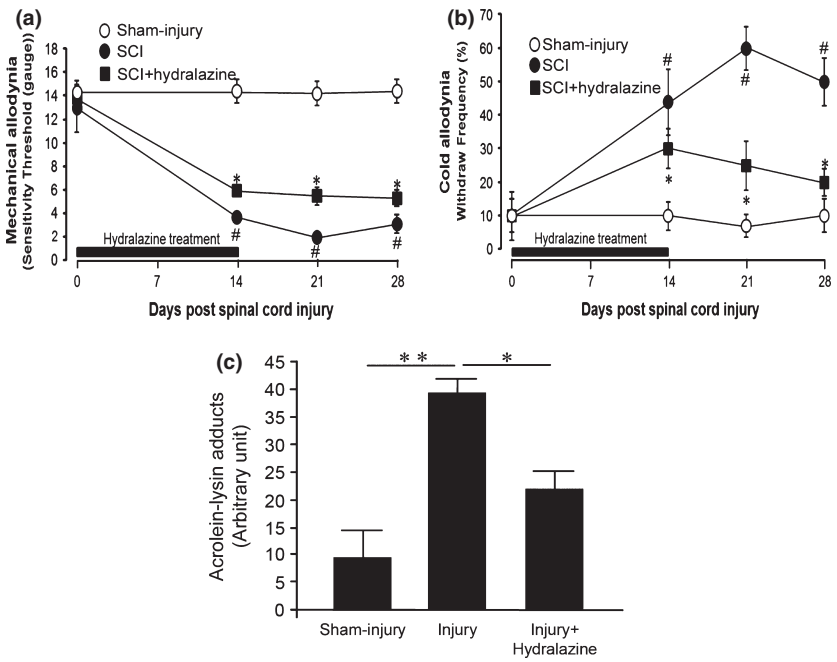

Fig. 5 Daily hydralazine injections for 14 days after thoracic spinal cord injury (SCI) attenuate behavioral changes in response to mechanical and cold stimuli for at least 28 days. At day 0, before SCI, there was no difference in mechanical and cold hyperreflexia assessments in sham-injured control, SCI alone, and SCI in combination with hydralazine. However, on days 14–28 post-injury, SCI alone rats displayed a significantly increased level of both mechanical and cold hyperreflexia. (a, b; #$p < 0.05$ when compared to the sham-injured control). This increased display of presumptive pain behavior was significantly attenuated with the addition of hydralazine (*$p < 0.05$ when compared to SCI alone). (c) Bar graph displaying the elevation of the acrolein-lysine adduct level and its attenuation by hydralazine 1 day after SCI. ($n = 6$ in each condition). (*$p < 0.05$, **$p < 0.01$, ANOVA). All data (a–c) were expressed in mean ± SEM.

Discussion

This study demonstrates that spinal cord contusion injury leads to the elevated levels of the oxidant, acrolein, for at least 2 weeks within the spinal cord at or near the injury site. This injury-induced formation of acrolein correlates with the onset of thermal and tactile-provoked pain behavior. Observations herein suggest that SCI induces both a decrease in the

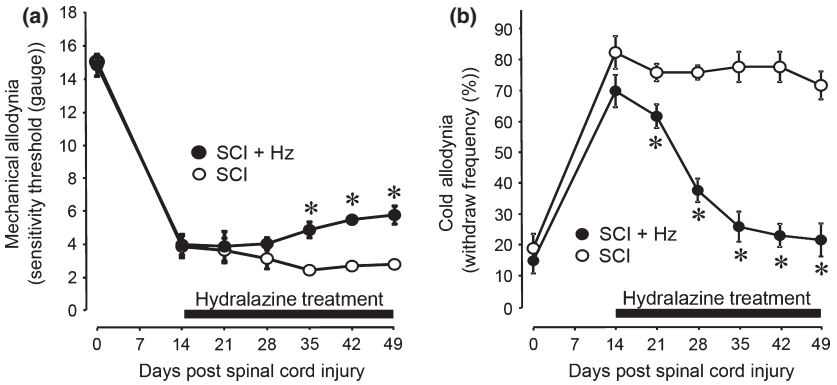

Fig. 6 Effective attenuation of thoracic spinal cord injury (SCI)-induced behavioral responses following delayed hydralazine administration in rats. The time course of changes in behavioral responses including SCI-induced mechanical hyperreflexia (a) and cold hyperreflexia (b) were effectively diminished following delayed hydralazine administration (daily injection days 14–49) in SCI rats. At day 0 (before SCI) and day 14 (2-week post-SCI), no difference was observed in either mechanical or cold hyperreflexia between SCI alone or SCI+Hz treatment group. However, hydralazine significantly attenuated the mechanical hyperreflexia in the SCI group starting 35-day post-SCI and continuing until the end of experiment. Likewise, hydralazine also significantly attenuated cold hyperreflexia starting 21-day post-SCI and continued through the remainder of the experimental period. Unpaired Student's *t*-test was used for statistical analysis. (*$p < 0.05$ when compared to SCI group, $n = 4$ for all groups). All data were expressed as mean ± SEM.

CT for action potential generation and an increase in the total number of acrolein-sensitive sensory neurons. Moreover, the evidence that the direct exogenous administration of acrolein into rodent spinal cord increases both tactile and thermal hyperreflexia combined with the observation that acrolein exposure *in vitro* increases neuronal excitation in small and medium diameter sensory neurons strongly supports the role of acrolein as an endogenous pro-nociceptive agonist.

In this study, we observed elevated levels of acrolein which may mediate increased pain signaling following SCI by promoting hyperexcitabilty of lumbar DRG neurons. Although our analysis regarding elevated levels of acrolein was restricted to the thoracic spinal cord injury site, the circulation of the oxidant is likely to spread via subarachnoid space within the spinal cord compartment (Luo *et al.* 2005). Moreover, because of the permeability of the DRG capsule and associated dorsal roots there is a strong possibility that the release of acrolein because of CNS injury can directly affect function of sensory neurons (Devor 1999; Abram *et al.* 2006; Puljak *et al.* 2009).

The direct effects of acrolein on nociceptive neurons likely evoke inward calcium current through the TRPA1 receptor and may serve to activate phospholipase C signaling pathways (Bautista *et al.* 2006; Dai *et al.* 2007). Previous studies also suggest that the TRPA1 receptor may facilitate activity in pain pathways via different stimuli (Bautista *et al.* 2006; Lennertz *et al.* 2012; Zhao *et al.* 2012; Perin-Martins *et al.* 2013). Though a recent report implicates SCI-induced changes in TRPV1 as a possible influence on behaviorally hypersensitivity (Wu *et al.* 2013), the observed changes in neuronal sensitization following acrolein administration may occur without concurrent changes in TRPV1 sensitivity (Frederick *et al.* 2007; Yang *et al.* 2008; Andrade *et al.* 2011).

Little is known regarding changes in the expression of transient receptor potential channels post-injury. A number of these channels exhibit decreased mRNA expression within the DRG following peripheral nerve injury (Frederick *et al.* 2007; Staaf *et al.* 2009; Braz and Basbaum 2010). Whether these injury effects are dependent on the loss of the continuous transport of peripherally derived neurotrophins is currently unknown. Some evidence suggests that TRPA1 and TRPV1 expression is decreased following a variety of neuropathic pain models (Michael and Priestley 1999; Hudson *et al.* 2001; Fukuoka *et al.* 2002; Obata *et al.* 2005; Katsura *et al.* 2006; Staaf *et al.* 2009) while more recent observations suggest that changes in TRPV1 expression can occur long after initial SCI (Wu *et al.* 2013).

TRPA1 is considered a promising target for analgesic drugs. TRPA1 antagonists are known to be effective in blocking pain behaviors induced by inflammation. However, the degree to which these reagents confer analgesic relief in the SCI rodent model is unknown (Nagata *et al.* 2005; Xu *et al.* 2005; Eid *et al.* 2008; McGaraughty *et al.* 2010; Chen *et al.* 2011). In addition, the receptor antagonists that are effective for SCI pain will likely remain untested, as many of these compounds are not suited for use as an analgesic compound.

As an endogenous agonist of TRPA1, acrolein may prove to be the more suitable therapeutic target. It is well known that acrolein released during inflammation and trauma can alter several of cellular processes throughout various organ systems, including the nervous system (causes damage to proteins, lipids, DNA, edema, ulceration and necrosis) (Shi

et al. 2002, 2011b; Bjorling *et al.* 2007; Hamann and Shi 2009; Leung *et al.* 2011). Based on the diffusive nature of acrolein, coupled with its stable and extended presence in the body, acrolein may very well serve as inflammation-associated compound that contributes to the chronic nature of post-SCI thermal and tactile hyperreflexia. Beyond the ability of acrolein to inflicting myelin damage and axonal degeneration in the central nervous system, little is known as to what degree acrolein contributes to the pathogenesis of chronic inflammation in the contused spinal cord, causing the need for further study and investigation (Shi *et al.* 2011b).

The involvement of acrolein in neuropathic pain was further assessed by treating rodents with hydralazine, a known scavenger of acrolein. We found that hydralazine treatment at the time of injury diminished both the presence of acrolein-lysine adducts and produced a modest effect on behavioral sensitivity to tactile and thermal stimuli. Similar behavioral effects were evident when the hydralazine treatment was delayed 14 days. However, we cannot rule out other effects of hydralazine on the associated nervous system. Though hydralazine is known to increase cGMP in smooth muscle leading to arteriole relaxation, the degree to which this drug directly alters sensory neurons is unknown. Complicating the actions of hydralazine in the nervous system is the finding by Song and colleagues that sensory neurons *in vitro* respond to agonists of cGMP-PKG signaling pathways with further increases in excitability (Song *et al.* 2006; Zheng *et al.* 2007). Based on these findings, one might predict that hydralazine should enhance both hyperreflexic behaviors in the SCI rodent and nociceptive hypersensitivity in sham injury control animals. In contrast, we failed to observe augmentation of hyperreflexic behavior in either treatment condition. Moreover, that the drug easily crosses the blood brain barrier during acute hypertensive encephalopathy might suggest the necessity of disease or injury processes for penetration of hydralazine into the intracranial compartments (Overgaard and Skinhoj 1975). Therefore, the actions of hydralazine on acrolein within the injured spinal cord and the associated reduction in hyperreflexic behavioral changes have to be balanced against presumptive pro-nociceptive effects of the drug on nociceptive sensory neurons.

There is also the possibility that other transient receptor potential channels may be sensitive to acrolein and contribute to SCI-induced neuropathic pain. TRPM8 of the transient receptor potential family has been shown to be activated by both cold and the cold-mimetics, menthol and icilin, *in vitro* (McKemy *et al.* 2002; Peier and Patapoutian 2002; Liu *et al.* 2013), agonists that have also been shown to bind to TRPA1 (Story *et al.* 2003). TRPM8 has been shown to play a significant role in injury-evoked hypersensitivity to cold following sciatic nerve injury or tissue inflammation (Colburn *et al.* 2007) and unlike TRPA1, plays a key role in cold pain (Knowlton *et al.* 2010). Recent evidence indicates that TRPM8 mRNA in L1-L6 DRGs is significantly lower than that of TRPA1 mRNA under naïve conditions (Vandewauw *et al.* 2013). In addition, acrolein does not bind to TRPM8, suggesting that the effects of hydralazine act by reducing the effect of acrolein-induced neuronal hyperexcitability via TRPA1 and not TRPM8.

Previous reports have supported the idea that SCI can have surprisingly strong effects on the excitability of nociceptor cell bodies (Bedi *et al.* 2010). *In vivo* and *in vitro* recordings of nociceptive C and Aδ fibers and dissociated DRG, respectively, showed that SCI dramatically increased the incidence of spontaneous activity generated within L4 and L5 DRG (50–70% of neurons recorded) far below the injury level. In addition, it has been reported that knocking down the expression of the nociceptor-specific Na+ channel, NaV1.8, which is important for the expression of nociceptor spontaneous activity, largely eliminates SCI-induced nociceptor spontaneous activity *in vitro* and greatly reduces behavioral hypersensitivity to both mechanical and thermal test stimulation applied *in vivo* (Yang *et al.* 2012). Together, these findings combined with the present results suggest that observed central alterations following SCI may be driven in large part by chronic hyperexcitability of primary sensory neurons.

Conclusions

Our data thoroughly support acrolein as a potent pro-nociceptive agonist that enhances neuronal sensitization of cells derived from SCI rodents when compared with those from control groups. Although the manner in which acrolein influences the sensitivity of DRG nociceptive neurons via TRPA1 or its exact contribution to neuropathic pain behavior following SCI is unknown, acrolein remains a continuous therapeutic target. This is because even if acrolein's initially elevated levels return to normal, the DRG neurons may remain chronically hypersensitive to its effects because of persistent up-regulation of TRPA1 after SCI. This clearly suggests that the window of analgesic capability based on the anti-acrolein strategy may not be limited to the initial stage when acrolein is elevated, but could also be effective in later or even chronic stages of neuropathic pain.

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Competing interests

The authors declare no competing financial interests.

Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Figure S1. Spinal cord injury (SCI) decreases the current threshold for action potential generation in small and medium diameter sensory neurons by fourteen days following injury.

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