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Enhancing KCNQ channel activity improves neurobehavioral recovery after spinal cord injury

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Running Title: Early application of retigabine to treat SCI dysfunction

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List of Nonstandard Abbreviations

BBB: Baso, Beattie, Bresnahan

CPP: Conditional place preference

EC: Eriochrome Cyanine

GFAP: Glial fibrillary acidic protein

Kv: Voltage-gated potassium channels

MAPK: Mitogen-activated protein kinase

SA: Spontaneous activity

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SCI: Spinal cord injury

SD: Sprague-Dawley

pSTAT3: Phosphorylated signal transducer and activator of transcription 3

TRPV1: The transient receptor potential cation channel subfamily V member 1

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ABSTRACT

Spinal cord injury (SCI) usually leads to acute neuronal death and delayed secondary degeneration, resulting in sensory dysfunction, paralysis, and chronic pain. Excessive excitation is one of the critical factors leading to secondary neural damage initiated by various insults. KCNQ/Kv7 channels are highly expressed in spinal neurons and axons, and play an important role in controlling their excitability. Enhancing KCNQ channel activity by using its specific opener retigabine could thus be a plausible treatment strategy to reduce the pathology following SCI. We produced contusive SCI at T10 in adult, male rats, which then received 10 consecutive days' treatment with retigabine or vehicle starting 3 hours or 3 days after contusion. Two different concentrations and two different delivery methods were applied. Delivery of retigabine via Alzet osmotic pumps, but not intraperitoneal injections 3 hours after contusion promoted recovery of locomotor function. Remarkably, retigabine delivery in both methods significantly attenuated the development of mechanical stimuli-induced hyperreflexia and spontaneous pain although no significant difference in the thermal threshold was observed. While retigabine delivered 3 days after contusion significantly attenuated the development of mechanical hypersensitivity and spontaneous pain, the locomotor function is not improved by the delayed treatments. Finally, we found that early application of retigabine attenuates the inflammatory activity in the spinal cord and increases the survival of white matter following SCI. Our results suggest that decreasing neuronal excitability by targeting KCNQ/Kv7 channels at acute stage aids the recovery of locomotor function and attenuates the development of neuropathic pain after SCI.

SIGNIFICANCE STATEMENT

Several pharmacological interventions have been proposed for SCI treatment, but none have been shown to be both effective and safe in clinical trials. Necrotic neuronal death and chronic pain often are the cost of pathological neural excitation after SCI. We show that early brief application of retigabine could aid locomotor and sensory neurobehavioral recovery following SCI, supporting the use of this drug in the clinic to promote motor and sensory function in SCI patients.

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INTRODUCTION

The spinal cord that contains neurons and major bundles of axons is critical for transmitting impulses between the brain and the peripheral nervous system. However, the vertebrate protected spinal cord can be damaged by vehicle accidents, sports-related accidents, war injuries, and tumors, which results in 17,000 acute spinal cord injury (SCI) cases every year and approximately 300,000 sustained traumatic SCI patients in the US (Rabinstein, 2018). SCI usually results in acute neuronal death and secondary degeneration of spinal tissues, followed by a chronic stage where cavities, cysts, and scar tissue are formed, leaving the individual with paralysis, autonomic problems, sensory dysfunction, and chronic pain for the rest of their life (Anderson and Hall, 1993). Treatment following SCI offers a major biomedical challenge. It is well known that some of the pathological consequences in the spinal cord following initial injury are caused by multiple factors that induce secondary degeneration. Many ascending sensory and descending motor tracts in the spinal cord often remain intact following initial traumatic injury (Anderson and Hall, 1993). The initial continuity of the residual white matter together with knowledge of the factors involved in secondary injury processes suggest that early application of pharmacological treatments that counteract the secondary cascade could minimize their pathological consequences, and thus promote the survival and functional recovery of spinal tissue.

A widespread event in both initial and secondary injury following SCI is cell depolarization and consequent intracellular Ca^{2+} overloading (through voltage-gated Ca^{2+} channels and ligand-gated Ca^{2+}-permeable ion channels) (Ross et al., 1999; Hall and Springer, 2004), which causes excitotoxicity and expands the lesion both horizontally and rostrocaudally to cause further neuronal degeneration and axonal demyelination. For example, the blockade of persistent inward

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Na^+ currents and glutamate release with riluzole provides a powerful neuroprotective effect in SCI animals (Lips et al., 2000; Schwartz and Fehlings, 2001; Nogradi et al., 2007). L-type Ca^{2+} channels are expressed in both spinal cord motor neurons and subgroups of interneurons (Morisset and Nagy, 1996; Carlin et al., 2000; Collins et al., 2001), which modulate neuronal excitability directly (depolarizing neurons) or indirectly (enhancing neurotransmission). Nimodipine, an L-type Ca^{2+} channel blocker, has been demonstrated to promote spinal cord function of SCI rats (Fehlings et al., 1989). Thus, minimizing persistent depolarization is a plausible strategy to treat SCI-induced dysfunctions.

KCNQ channels belong to the Kv7 subfamily (Kv7.1-Kv7.5) (Jentsch, 2000), and Kv7.2-Kv7.5 are present in sensory neurons, spinal cord interneurons, motor neurons and various axons (Alaburda et al., 2002; Passmore et al., 2003; Devaux et al., 2004). They play an important role in stabilizing resting membrane potentials. Importantly, retigabine (ezogabine), a specific KCNQ channel activator (selectively targeting Kv7.2-Kv7.5 channels) and approved by the FDA to treat partial epilepsies, hyperpolarizes primary afferent fibers (Rivera-Arconada and Lopez-Garcia, 2005) and reduces the transmission of Aδ and C-fiber activity to dorsal horn neurons (Passmore et al., 2003). Also, retigabine decreases sensory Aδ and C-fiber discharges induced by heat stimulation in an isolated rat skin-nerve preparation. Conversely, a KCNQ/Kv7 channel inhibitor, XE991, significantly increases the peripheral Aδ discharge produced by both thermal and mechanical stimulation *in vivo* (Brown and Passmore, 2009).

Recent work shows that chronic spontaneous activity (SA) occurs in primary nociceptors of rats after SCI (Bedi et al., 2010). Delivery of retigabine causes a reversible hyperpolarization, suppresses SA (both *in vivo* and *in vitro*), and relieves signs of chronic SCI pain (Wu et al., 2017). The KCNQ channel activator, retigabine, dose-dependently prevents both serum

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withdrawal-induced and NMDA-induced neurodegeneration *in vitro* (Boscia et al., 2006). Retigabine pretreatment affords neuroprotective effects for chemotherapy-induced peripheral neuropathy (Nodera et al., 2011; Li et al., 2019). Also, open KCNQ channels protect neurons from cerebral ischemia (Rekling, 2003). Since excessive neuronal excitation is an important factor for secondary degeneration after SCI, reducing the activity of neurons by opening KCNQ/Kv7 channels may protect spinal neurons and axons from degeneration after SCI, thereby promoting recovery of motor and sensory function. In this study, we report that repeated application of retigabine to open these channels at the acute stage promotes neurobehavioral recovery following SCI.

MATERIALS AND METHODS

All procedures conformed to the guidelines of the International Association for the Study of Pain and followed the Guide for the Care and Use of Laboratory animals. The protocols were approved by the animal care and use committee of the University of Texas Medical Branch at Galveston. Male, adult, Sprague-Dawley rats (200-300g) were housed two per woodchips-bedding cage in an animal facility with a controlled environment (21 ± 1°C, 12-hour dark/light reversed cycle), and had free access to standard rat chow and drinking water. The rats were purchased from Charles River and a 1-week habituation period was applied before the experiment.

Spinal cord injury

SCI in humans are generally resulted from contusion of the spinal cord. Thus, a spinal cord contusion model was used. Considering the variability in spinal cord damage induced by

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different contusion models (Young, 2002), contusion was performed using Infinite Horizon impactor (Precision Systems & Instrumentation, Lexington, KY), which creates a reliable contusive injury to the exposed spinal cord by rapidly applying a force-defined impact (Scheff et al., 2003). Animals were anesthetized with a mixture of acepromazine (0.75 mg/kg), xylazine (20 mg/kg), and ketamine (80 mg/kg) followed by laminectomy and moderate spinal contusion (150 kdyne with 1 s dwell time) at T10. Sham animals underwent a laminectomy without spinal impact. The muscles were sutured over the spine before stapling the skin flaps with wound clips. SCI/laminectomy animals were then returned to their cages that were placed on heating pads (~37 °C). Post-injured animals received twice daily injections of analgesic (buprenorphine; 0.02 mg/kg, i.p.) for 5 days and prophylactic antibiotics (Baytril, 2.5 mg/kg, i.p.) for 10 days. The bladder was manually evacuated twice daily until recovery of bladder function. Rats were checked for any behaviors of spontaneous pain, which include extensive grooming, marked inactivity, or autotomy. Any animals exhibiting severe signs of spontaneous pain were euthanized immediately.

Delivery of retigabine

Two delivery methods were used in this study. Considering that the half-life of retigabine is 8-11 hrs (Luszczki, 2009), SCI rats were given retigabine or vehicle twice daily by intraperitoneal injection (i.p.) for 10 consecutive days starting 3 hrs or 3 days after contusion, or received constant infusion via a mini-osmotic pump. Our previous study indicates that 10 mg/kg retigabine via intraperitoneal injection is effective in attenuating SCI-induced chronic pain. While the threshold concentration for inhibiting neuronal activity via i.p. injected retigabine is controversial (Xu et al., 2010; Hayashi et al., 2014), at least one study indicates that 2.5 mg/kg

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retigabine (i.p.) inhibits CFA-induced mechanical allodynia (Xu et al., 2010). Thus, the concentrations at 10 mg/kg and 3 mg/kg were used for i.p. injection. 750 μg/kg/hr for osmotic pump was used because the amount is close to 10 mg/kg i.p. injections and easy for calculation. Mini-osmotic pumps (Alzet 2002, 0.5 μl/h; Alzet Corp., Cupertino, CA) were filled with 0.9% saline (vehicle) or retigabine in 0.9% saline with syringe. The pre-filled osmotic pumps were then placed in saline at 37 °C for overnight. Pre-loaded and primed pumps were then implanted subcutaneously in the flank followed spinal cord contusion/laminectomy. Retigabine or saline was delivered by pumps 3 hours or 3 days after impact for 10 days. Retigabine concentration in the blood reach the peak in 90 mins after i.p. injection (Luszczki, 2009). It will take more than 3 hrs for the osmotic minipumps to deliver (750 μg/kg/h) accumulated amount of 2.5 mg/kg retigabine. In order to quickly establish effective concentration in the time-course that is comparable to i.p. injection, a booster dose (3 mg/kg retigabine, i.p.) was administered immediately before perfusion.

Behavioral tests

Reflex sensitivity tests: Standard five-day sequence of tests for hindlimb reflex sensitivity were performed before impact and then after retigabine/vehicle treatments, as described previously (Bedi et al., 2010). The reflex tests were performed under red light. The experimenters collected reflex data were blinded to any drug treatment. Before each behavioral test, animals were allowed to habituate for 20-min in each of the testing chambers over a 5-day period. Below-level thermal sensitivity (heat) was tested using the Hargreaves radiant heat method (IITC Plantar Analgesia Meter, Woodland Hills, CA) in which the withdrawal latency of the hindpaws was measured. To prevent possible tissue injury, the stimulus was terminated if no withdrawal

occurred within 20 s. Both hindpaws were tested. The test sequence was performed 3 times, at 20 min intervals. The mean of six latency measurements (3 from each hindpaw) was used for each data point for each animal. A series of calibrated von Frey filaments (Stoelting, Wood Dale, IL) were used to test below-level mechanical sensitivity by stimulating the glabrous surface of the hindpaws (Hulsebosch et al., 2000). Thresholds were determined with the "up-down" method (Chaplan et al., 1994). Each hindpaw received only one test series.

Conditioned place preference （CPP） test for ongoing pain: CPP tests were performed as previously described (Yang et al., 2014). Briefly, rats (both SCI animals accepted brief vehicle- and retigabine-treatment) were allowed to adjust to the CPP device on the day one following a 3-day conditioning phase in which animals received twice-daily conditioning injections. In analgesic session, the rats received conditioning anesthetic, retigabine (10 mg/kg, i.p.). Five minutes after retigabine injection, animals were kept in the non-preferred white chamber for one hour. In the other daily session (non-analgesic session), rats received identical amount of vehicle (saline, i.p.). Five minutes after vehicle injection, animals were kept in the preferred black chamber for one hour. One day after the conditioning session, animals without any injection were introduced into the central open gray chamber. The time that the animal spent in each of the three chambers (gray, white, and black) was recorded for 15 min. Animals spent more time in the white chamber (paired with analgesic injection during conditioning phase) than that in the black chamber (paired with saline injection during conditioning phase) during the testing phase were considered having chronic spontaneous ongoing pain (i.e., if the time in white minus time in black was positive).

Basso, Beattie, and Bresnahan (BBB) locomotor scoring: To detect the effects of SCI on locomotor function, the 21-point Basso, Beattie, Bresnahan (BBB) locomotor test was used

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(Basso et al., 1995). Rats were placed in an open field container, their locomotor behavior was observed and scored in white light (Basso et al., 1995). Testing was performed daily for the first 5 days and then weekly thereafter for 5-7 weeks after contusion. Animals with a BBB score of 1 or more than 1 at day one after contusion were excluded from SCI group.

Horizontal ladder: One week before injury, a training session was performed. Rats were trained daily to run across a horizontal ladder that was 1-m-long with a testing field of 0.8 m. The testing field contained 10 randomly allocated rungs that were spaced 3-8 cm apart. Each session consisted of 3 ladder crossings. On the final day of the training session, tests were videotaped. The videotapes were viewed in slow motion so that the total number of hindlimb misses and footslips during the course can be quantified and a basal score can be assigned to each animal tested. If the entire paw going below the rung during the testing, a score of 1 was assigned. This test was then performed once a week for 5 weeks, starting on day 14 after contusion.

Western Blotting

After behavioral tests (42 days after contusion), animals were deeply anesthetized with Beuthanasia (Merck, Kenilworth, NJ) and perfused with ice-cold PBS. The L4 and L5 of the spinal cords from each rat were removed and immediately placed in a 1.5 ml Eppendorf tubes on dry ice. Tissues were homogenized in 500 µl of lysis buffer (RIPA, Teknova) containing a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). After homogenization, samples were sonicated 3 times (10 s pulses), and centrifuged at 14,000 rpm for 10 min at 4 ^{o}C. The protein concentration of lysates was determined by the BCA assay (Pierce BCA Protein Assay Kit). Samples were prepared for SDS-PAGE (Bio-Rad, 4-20% Tris-HCl) by 1:1 dilution with Laemmli sample buffer, and 30 µg of protein was loaded in each well. After electrophoresis, the

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gel was transferred to a PVDF membrane and blocked with 10% nonfat dry milk in PBS + 0.1% Tween 20 prior to incubation with antibody against GFAP (Millipore, USA; AB5541), Iba-1 (JUJIFILM Wako Chemicals, 016-20001), pSTAT3 (Cell Signaling Technology, MA, 9211), p38 (Cell Signaling Technology, MA, 9212), and β-actin (Abcam, MA; ab6276) overnight at 4 ^{o}C. The membrane was incubated with HRP-conjugated anti-rabbit or anti-mouse IgG (Jackson ImmnuoResearch, PA) for 1 hour at room temperature and developed using the ECL kit (Pierce). Protein expression was quantified by optical density using Image J software (NIH). Color molecular weight standards were run on each gel and β-actin was detected as a loading control.

Eriochrome cyanine (EC) histochemistry

Iron-eriochrome cyanine R (EC) was performed on spinal cord sections to differentiate gray and white matter using a protocol adapted from previous published methods (Rabchevsky et al., 2001; James et al., 2011). After all behavior tests (42 days after contusion), animals were euthanized with Beuthanasia (Merck, Kenilworth, NJ) and perfused with cold Phosphate-buffered saline (PBS) followed by 4% paraformaldehyde solution (PFA; Sigma Aldrich Co., St. Louis, MO). The spinal cords were then extracted (~10 mm) with the lesion site located centrally and post-fixed in 4% PFA for overnight. Tissues were then incubated in 30% sucrose at 4 °C for 24 hours. The spinal cords at lesion site were embedded in cryo-embedding media (OCT, Sakura Finetek, Torrance, CA) and then stored at -80°C. The tissues were cut into transverse sections at 20-μm thicknesses and were mounted on a series of positive charge slides with 200-μm intervals between sections. The section of each animal with the largest cavity was defined as the lesion epicenter. The quantification of the lesion volume was analyzed from 3 mm caudal/rostral to the

epicenter. Sections were dehydrated, cleared, rehydrated, and then stained. Stained sections were viewed using a Nikon microscope and quantified using NIS Elements software.

Data analysis

Animal numbers in each experiment were determined by power analysis, published reports, and past experience. All data were presented as mean ± S.E.M. Data was analyzed with Prism 8.0 (Graphpad, La Jolla, CA) and Sigmaplot 14 (Systat software, San Jose, CA). The data were first tested for normality of distribution and homogeneity of variance. If some data do not meet these requirements, non-parametric equivalent analyses were used. Repeated measures two-way ANOVA followed by Sidak's multiple comparison tests, one-way ANOVA with repeated measures followed by Bonferroni's *post-hoc* tests, or unpaired t-test (two-tailed) were used to analyze the significance difference among different animal groups. BBB scoring were compared between groups on days -1, 1, 2, 3, 7, 14, 21, 28, 35, and 42. The hindlimb footslips were compared between groups on days -1, 14, 21, 28, 35, and 42. The α level is 0.05 for all statistical tests, $P < 0.05$ was deemed statistically significant. The n in all experiments is the number of animals used. Statistically significant differences were illustrated in each figure (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

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RESULTS

Brief retigabine delivery at acute stage attenuates the development of mechanical hyperreflexia and spontaneous ongoing pain following SCI

It is reported that retigabine reverses established SCI-induced reflex hypersensitivity (Wu et al., 2017). We then observed the effect of early brief delivery of retigabine (3 hrs after contusion for 10 consecutive days) on the development of pain-like behavior following SCI. Thermal and mechanical sensitivity of hindpaws were tested. Remarkably, retigabine significantly mitigated the development of mechanical stimuli-induced hindlimb hyperreflexia (**Fig. 1A)** in all delivery methods (i.p., 3 mg/kg, twice daily and Alzet osmotic pump, 750 μg/kg/hr) although thermal (heat) threshold values between retigabine and vehicle groups were not altered (**Fig. 1B**). Spontaneous ongoing pain also occurs in SCI patients, we thus performed the conditioned place preference test (CPP) 35 days after SCI to determine if retigabine alters the development of SCI-induced spontaneous ongoing pain as compared to their vehicle counterparts (Yang et al., 2014; Wu et al., 2017). After conditioning phase in which the conditioned analgesic treatment was paired with placement of animals in the innately less-preferred white chamber, the SCI animals received vehicle, but not SCI animals received retigabine via osmotic pumps starting 3 hrs after spinal cord contusion for 10 consecutive days, spend more time in the white chamber during testing phase (**Fig. 1C**). These results indicate that brief delivery of retigabine at acute stages of SCI mitigates the development of signs of chronic pain.

Early osmotic pump delivery, but not intraperitoneal injection, of retigabine promotes both BBB score and horizontal ladder performance after SCI

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Locomotor behavioral tests are important tools for evaluating the therapeutic efficacy of medicine after SCI. The effect of retigabine on locomotor function following SCI was observed at first. Both BBB (Basso, Beattie, and Bresnahan) 21-point open-field locomotion score and horizontal ladder test were used to evaluate the locomotor function of retigabine- and vehicle-treated SCI rats. Retigabine was delivered 3 hours after contusion for 10 consecutive days. Two different concentrations (3 mg/kg and 10 mg/kg, i.p. twice daily) were used. Neither 3 mg/kg (**Fig. 2A**) nor 10 mg/kg (**Fig. 2B**) retigabine showed a significant effect on BBB scoring. However, the hindlimb footslips tested with horizontal ladder shown significant improvement at some later time points after contusion (**Fig. 2C** and **2D**).

Considering that intraperitoneal injection of retigabine does not maintain the blood concentration of this medicine at a stable level, which probably diminishes its neuronal protective effect *in vivo*, we thus applied retigabine (750 μg/kg/hr) with the Alzet osmotic mini-pump starting 3 hours after contusion for 10 days, which maintained a stable retigabine concentration in animals as comparing to intraperitoneal injection. Different from outcomes after intraperitoneal injection, retigabine delivered via osmotic pumps (750 μg/kg/hr) promoted recovery of locomotor function that was measured with both BBB scoring (**Fig. 3A**) and horizontal ladders (**Fig. 3B**). These data strongly suggest that consistently decreasing neuronal excitability is important for the recovery of the locomotor functional after SCI.

Delayed application of retigabine (3 days after contusion) alleviates the development of chronic pain, but not motor dysfunction after SCI

We then delayed the application of retigabine (3 days after contusion) to see whether it also improves motor function after SCI and prevents the development of post-SCI pain. Retigabine (750 μg/kg/hr) was delivered for 10 consecutive days by an Alzet osmotic pump to SCI rats starting 3 days after contusion. The effects of retigabine on locomotor function following SCI were observed. Both the BBB open-field locomotion score and horizontal ladder test were performed to evaluate locomotor function in retigabine- and vehicle-treated SCI rats. Retigabine has no significant effect on BBB scoring (**Fig. 3C**) and hindlimb footslips tested with the horizontal ladder as compared to their vehicle-treated counterparts (**Fig. 3D**).

While motor function was not improved by delayed retigabine treatment via osmotic pump (750 μg/kg/hr, starting 3 days after contusion for 10 days), the development of mechanical hypersensitivity after SCI was attenuated. As compared to the vehicle-treated SCI animals, the hindpaw withdrawal threshold to mechanical stimulation (*von Frey*), but not hindpaw withdrawal delay to heat, was obviously higher in the retigabine-treated SCI animals 28 days after contusion (**Fig. 4A** and **B**). We also tested the spontaneous pain in SCI rats treated with delayed retigabine/vehicle. CPP test was performed 35 days after SCI. As compared to the vehicle-treated SCI animals, the development of preference for the conditioned analgesic injection paired white chamber was significantly attenuated by the delayed brief retigabine treatment (**Fig. 4C**). These results indicate that delayed administration of retigabine after SCI can still mitigate the development of SCI chronic pain.

Early brief delivery of retigabine after contusion prevents the activation of glial cells in the spinal cord.

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Glial cells are activated after spinal cord injury in the at-level, below-level and above-level of the lesion site of the spinal cord (Hains and Waxman, 2006; Carlton et al., 2009). We found that the expression level of GFAP and pSTAT3 at the lesion site of the spinal cord in retigabine (750 μg/kg/hr, osmotic pump, starting 3 hrs after contusion for 10 days.)-treated SCI groups were significantly decreased as compared to those of the vehicle-treated SCI groups (**Fig. 5A** and **B**). However, we did not observe a significant difference in the expression level of Iba-1, a marker of microglia, in the lesion site, between retigabine- and vehicle-treated groups (**Fig. 5C**). Considering that the direct role of Iba-1 in chronic pain genesis is unclear, we thus used an additional marker that is functionally linked to neuropathic pain development, phosphorylated-P38 MAPK (Jin et al., 2003). Again, no significant differences in P38 MAPK expression levels were found between brief retigabine and vehicle-treated animals (**Fig. 5D**).

Early brief delivery of retigabine after contusion attenuates the necrosis of the spinal cord neurons.

Morphological evidence was also collected histologically by measuring lesion volume in the spinal cord. The most important outcome was increased survival of spinal neurons and axons. Based on the result from motor function tests, we emphasized the data from animals treated with retigabine via osmotic pump 3 hrs after contusion for 10 days. We measured the volume of spared white matter, and gray matter at the injury site by eriochrome cyanine (EC) staining. As shown in **Fig. 6**, we observed more surviving spinal tissue in the retigabine-treated animals after spinal contusion as compared to that from the vehicle-treated rats. The areas of spared white matter were significantly preserved in the retigabine (SCI + retigabine) group than in the control (SCI + vehicle) group. However, no significant differences in spared gray matter was found

between brief retigabine- and vehicle-treated animals (**Fig. 6**). These results indicate that early retigabine treatment decreases the degeneration of spinal tissues following SCI.

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DISCUSSION

Injury to the spinal cord leads to a number of deficits, including motor dysfunction and chronic pain. A widespread event in both initial and secondary injury following SCI is neuronal depolarization (Ross et al., 1999; Hall and Springer, 2004), which causes cytotoxicity that lead to further neuronal degeneration, axonal loss, and axonal demyelination. Decreasing neuronal excitability by blocking persistent Na^+ currents and glutamate release with riluzole decreases locomotor dysfunction and pain after SCI (Lips et al., 2000; Schwartz and Fehlings, 2001; Nogradi et al., 2007). KCNQ ion channels are widely expressed in the primary sensory neurons, spinal cord motor neurons and interneurons and axons (Alaburda et al., 2002; Passmore et al., 2003; Devaux et al., 2004). They play an important role in stabilizing resting membrane potentials. Recent studies indicate that decreasing neuronal excitability at early stages by targeting KCNQ channels attenuates the development of chemotherapy-induced neuronal degeneration and chronic pain (Li et al., 2019), amyotrophic lateral sclerosis-induced motor neuron death (Wainger et al., 2014), and stroke-induced dysfunction (Bierbower et al., 2015; Vigil et al., 2019). The current study shows that neutralizing neuronal excitability by pharmacologically enhancing the activity of KCNQ channels with retigabine could be a useful strategy for attenuating the development of SCI-induced functional and pathological alterations. Enhanced neuronal activity has been associated with elevations in cytosolic calcium concentration induced by calcium influx through voltage sensitive calcium channels (VSCCs) and calcium release from intracellular stores. Although our previous study indicates that the Kv7 channel expression and function in DRG neurons are unchanged after SCI (Wu et al., 2017), we cannot exclude the possibility that the expression and function of Kv7 in the spinal cord motor neurons and interneurons are altered since Ca^{2+}-calmodulin suppresses the trafficking and/or

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function of Kv7 channels (Alaimo and Villarroel, 2018), which raises the possibility of positive feedback loops driving neuronal over-excitation. The increased cytosolic Ca^{2+} concentration lead to multiple cellular pathological alterations. For example, increased intracellular Ca^{2+} triggers mitochondrial Ca^{2+} overloading, which exaggerate neuronal functions by jeopardizing mitochondrial respiration (Llorente-Folch et al., 2015), producing reactive oxygen species (Adam-Vizi and Starkov, 2010), impairing mitochondrial dynamic via mitophagy (Brady et al., 2007), and inducing apoptosis (Giorgi et al., 2012). These alterations thus ultimately result in the injury-induced secondary degeneration in the spinal cord. In addition, maintaining neuronal membrane potential alone during their enhanced activity consumes more than 90% of energy produced by the cell (Erecinska et al., 1994; Erecinska and Silver, 1994). Neuronal over-excitation may thus lead to energy exhaustion and degeneration because other energy-dependent processes that are critical for cell maintenance, including molecular transportation and ionic exchanging, will suffer the torments of hunger. Thus, although neuronal injury can be induced by difference causes in different locations, neuronal hyperpolarization after neuronal injury is likely a shared mechanism that induces neuronal degeneration and plasticity.

Both BBB score and ladder tests were used to detect the motor function of SCI animals. However, their outcomes were not consistent. Ladder tests showed a better recovery in motor function as compared to those from BBB scoring, especially when the retigabine was applied via an osmotic mini-pump that can maintain the drug concentration at a stable level. It has been demonstrated that BBB scores show low correlation with fiber conductions, especially at the recovery stage in which BBB scores show no further improving but fiber conduction is still ameliorating (James et al., 2011). In contrast, there is a high correlation between ladder scores

and percentage of conducting fibers (James et al., 2011). Our histology study indicates that early application of retigabine protects white matter after SCI. Preserving even 5-10% of the axonal population could aid the recovery of locomotor and sensory function (Blight, 1983). Cell depolarization is an important contributor to secondary degeneration after SCI (Ross et al., 1999; Hall and Springer, 2004). Our data suggest that neuronal depolarization occurs at the early stage of the secondary degeneration since the locomotor outcomes resulted from early treatment (3 hours) are better than those from delayed treatment (3 days).

The hyperexcitability of primary nociceptors is critical for the initiation and maintenance of SCI-induced chronic pain (Bedi et al., 2010; Yang et al., 2014). As compared to the central nervous system, an effective vascular permeability barrier is lacking in the DRG (Hirakawa et al., 2004; Abram et al., 2006; Jimenez-Andrade et al., 2008), their cell bodies are likely to be exposed to the highest concentrations of peripherally applied retigabine, thereby the effect of retigabine on chronic pain is not that sensitive to the delivery routes and starting time.

Retigabine is used for both the preventive treatment following SCI and the CPP conditioning in this study. Our data indicate that early brief retigabine, but not delayed retigabine, attenuates the development of SCI-induced neurobehavioral dysfunction. The CPP tests were performed 4 weeks after contusion, the interference induced by conditional retigabine during CPP tests is thus minimal if there is any. Spinal cord injury induces both mechanical and thermal hyperreflexia (Bedi et al., 2010; Wu et al., 2013; Yang et al., 2014; Wu et al., 2017). Acute application of retigabine decreases established heat and mechanical hypersensitivity after SCI (Wu et al., 2017). However, brief application of retigabine at acute stage decreases only the development of

mechanical hypersensitivity, but not that of heat hypersensitivity after SCI. Several factors have been shown to be critical for the development of SCI-induced chronic pain, including TRPV1 ion channel plasticity in the primary sensory neurons (Wu et al., 2013), Nav1.3 overexpression and GABAergic alteration in the spinal cord dorsal horn neurons (Hains et al., 2003; Lu et al., 2008; Meisner et al., 2010), glial cell activation in the central nervous system (Hains and Waxman, 2006; Zhao et al., 2007; Detloff et al., 2008; Carlton et al., 2009), and descending antinociceptive serotonergic pathway disruption (Bruce et al., 2002). It is unknown to what extent these factors differentially contribute to mechanical and/or thermal hypersensitivity and how these different factors respond to retigabine treatment. Heat sensitivity is not always altered in parallel with the changes in mechanical sensitivity in chronic neuropathic pain models. For example, mechanical hypersensitivity, but not heat hypersensitivity, generally develops in paclitaxel-induced peripheral neuropathy that involves similar mechanisms (except microglial activation in the spinal cord) as SCI (Zheng et al., 2011; Burgos et al., 2012; Zhang et al., 2012; Li et al., 2015; Nashawi et al., 2016; Li et al., 2018; Li et al., 2019).

Spinal cord injury results in the invasion of cells of the immune system that can increase the excitability of neurons in the spinal cord (Detloff et al., 2008; Dulin et al., 2013). Although it is not clear as to how the immune system is activated after SCI, it has been demonstrated that at least neuronal hyperexcitability contributes to the activation of glial cells in the spinal cord (Xie et al., 2009), thus forming a positive loop that worsens the development of both locomotor dysfunction and chronic pain. Both astrocytes and microglial cells are activated after spinal cord injury in the at-level, below-level, and above-level of the lesion site of the spinal cord, which is linked to the development of SCI-induced neurobehavioral dysfunction (Hains and Waxman,

2006; Carlton et al., 2009). While the expression levels of astrocyte marker GFAP in the lesion site is significantly decreased after retigabine treatment, the changes in the expression levels of microglial markers Iba-1 and phosphorylated-P38 MAPK are not significant. One literature reports that SCI induces significant upregulation of the phosphorylated STAT3 and contributes to the astrogliosis in the spinal cord (Herrmann et al., 2008). Consistent with the changes in GFAP, the expression level of pSTAT3 in the spinal cord is decreased upon retigabine delivery, which suggests the treatment decreases the inflammatory activity. The early phase of cellular inflammation (10 days post injury) is correlated with impaired locomotor function, whereas the later phase of cellular inflammation (14 days to 180 days post injury) does not coincide with altered locomotor function after SCI (Beck et al., 2010). Our study indicates that decreasing neuronal excitability at early phases of SCI attenuates both the expression level of GFAP and inflammatory activity in the spinal cord, which might contribute to the improvement of locomotor function and chronic pain after SCI.

Author Contributions:

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Performed data analysis: Z. Wu, Q. Yang, F. Xie

Wrote or contributed to the writing of the manuscript: Q. Yang and Z. Wu

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Figure Legend

Figure 1. The effects of early brief application of retigabine (starting 3 hrs after contusion for 10 days) on development of pain behaviors after SCI. (**A**) Mechanical hypersensitivity of hindpaws was measured 28 days after initial traumatic spinal cord injury. Each dot in column represent one animal. P = 0.033, $t_{(21)}$ = 2.278 for groups with i.p. injection (3 mg/kg, twice daily); P = 0.0324, $t_{(24)}$ = 2.271 for groups with osmotic pump perfusion (750 μg/kg/hr). Two-tailed unpaired t-test. (**B**) Heat hypersensitivity of hindpaws was measured 28 days after initial traumatic spinal cord injury. Each dot in column represent one animal. P = 0.2058, $t_{(12)}$ = 1.338, for groups with i.p. injection (3 mg/kg, twice daily); P = 0.1112, $t_{(11)}$ = 1.732 for groups with osmotic pump perfusion (750 μg/kg/hr). Two-tailed unpaired t-test. *, $p<0.05$. (**C**) Spontaneous pain was evaluated with CPP tests 35 days after contusion. Rats were treated with retigabine via osmotic pump (750 μg/kg/hr). Each dot in column represent one animal. P = 0.0385, $t_{(8)}$ = 2.474, Two-tailed unpaired t-test. *, $P < 0.05$.

Figure 2. The effects of early repeated intraperitoneal administration of retigabine on the recovery of locomotor function after SCI. (**A**) BBB scoring was performed -1, 1, 2, 3, 7, 14, 21, 28, 35 and 42 days after contusion. SCI animals received twice daily either 3 mg/kg retigabine (i.p., twice daily) or identical vehicle 3 hrs after contusion for 10 consecutive days. Repeated measures two-way ANOVA followed by Sidak's multiple comparison test (treatment $F_{(1, 26)}$ = 0.1714, p = 0.6822; time $F_{(9, 234)}$ = 445.7, $P < 0.0001$; interaction $F_{(9, 234)}$ = 0.4473, P = 0.9081. Baseline, $P > 0.999$; 1 day, $P > 0.999$; 2 days, $P > 0.999$; 3 days, $P > 0.999$; 7 days, P = 0.9992; 14 days, P = 0.7635; 21 days, $P > 0.999$; 28 days, $P > 0.999$; 35 days, $P > 0.999$; 42 days, $P > 0.999$. (**B**) BBB scoring was performed -1, 1, 2, 3, 7, 14, 21, 28, 35 and 42 days after contusion.

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SCI animals received twice daily either 10 mg/kg retigabine (i.p., twice daily) or identical vehicle 3 hrs after contusion for 10 consecutive days. Repeated measures two-way ANOVA followed by Sidak's multiple comparison test (treatment $F_{(1, 18)} = 0.3431$, $P = 0.5653$; time $F_{(9, 162)} = 252.4$, $P < 0.0001$; interaction $F_{(9, 162)} = 0.6304$, $P = 0.7699$. Baseline, $P > 0.999$; 1 day, $P > 0.999$; 2 days, $P > 0.999$; 3 days, $P > 0.999$; 7 days, $P = 0.6373$; 14 days, $P > 0.999$; 21 days, $P > 0.999$; 28 days, $P > 0.999$; 35 days, $P = 0.9471$; 42 days, $P > 0.999$). (**C**) Horizontal ladder test was performed -1, 14, 21, 28, 35 and 42 days after contusion. SCI animals received twice daily either 3 mg/kg retigabine (i.p., twice daily) or identical vehicle 3 hrs after contusion for 10 consecutive days. Repeated measures two-way ANOVA followed by Sidak's multiple comparison test (treatment $F_{(1, 16)} = 4.432$, $P = 0.0516$; time $F_{(5, 80)} = 150.6$, $P < 0.0001$; interaction $F_{(5, 80)} = 2.545$, $P = 0.035$. Baseline, $P > 0.999$; 14 days, $P = 0.8821$; 21 days, $P = 0.9904$; 28 days, $P = 0.9994$; 35 days, $P = 0.0133$; 42 days, $P = 0.0333$). (**D**) Horizontal ladder test was performed -1, 14, 21, 28, 35 and 42 days after contusion. SCI animals received twice daily either 10 mg/kg retigabine (i.p., twice daily) or identical vehicle 3 hrs after contusion for 10 consecutive days. Repeated measures two-way ANOVA followed by Sidak's multiple comparison test (treatment $F_{(1, 13)} = 7.821$, $P = 0.0151$; time $F_{(5, 65)} = 145.9$, $P < 0.0001$; interaction $F_{(5, 65)} = 2.352$, $P = 0.0503$. Baseline, $P > 0.999$; 14 days, $P = 0.1383$; 21 days, $P > 0.9999$; 28 days, $P > 0.9999$; 35 days, $P = 0.0057$; 42 days, $P = 0.1423$). *, $P < 0.05$.

Figure 3. The effects of early and delayed osmotic pump delivery of retigabine on recovery of locomotor function after SCI. (**A**) BBB scoring was performed -1, 1, 2, 3, 7, 14, 21, 28, 35, and 42 days after contusion. SCI animals received either 750 μg/kg/hr retigabine or identical vehicle 3 hrs after contusion for 10 consecutive days. Repeated measures two-way ANOVA followed by

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Sidak's multiple comparison test (treatment $F_{(1, 17)} = 4.083$, $P = 0.0593$; time $F_{(9, 153)} = 219.1$, $P < 0.0001$; interaction $F_{(9, 153)} = 2.548$, $P = 0.0094$. Baseline, $P > 0.999$; 1 day, $P > 0.999$; 2 days, $P = 0.9967$; 3 days, $P = 0.8643$; 7 days, $P = 0.6936$; 14 days, $P = 0.0286$; 21 days, $P = 0.0402$; 28 days, $P = 0.0452$; 35 days, $P = 0.0482$; 42 days, $P = 0.7149$. (**B**) Horizontal ladder test was performed -1, 14, 21, 28, 35, and 42 days after contusion. SCI animals received either 750 μg/kg/hr retigabine or identical vehicle 3 hrs after contusion for 10 consecutive days. Repeated measures two-way ANOVA followed by Sidak's multiple comparison test (treatment $F_{(1, 11)} = 5.805$, $P = 0.0347$; time $F_{(5, 55)} = 54.12$, $P < 0.0001$; interaction $F_{(5, 55)} = 3.105$, $P = 0.0154$. Baseline, $P > 0.999$; 14 days, $P = 0.0148$; 21 days, $P = 0.0358$; 28 days, $P = 0.0408$; 35 days, $P = 0.0991$; 42 days, $P = 0.0451$). (**C**) BBB scoring was performed -1, 1, 2, 3, 7, 14, 21, 28, 35, and 42 days after contusion. SCI animals received either 750 μg/kg/hr retigabine or identical vehicle 3 days after contusion for 10 consecutive days. Repeated measures two-way ANOVA followed by Sidak's multiple comparison test (treatment $F_{(1, 11)} = 0.00807$, $P = 0.93$; time $F_{(9, 99)} = 270.7$, $P < 0.0001$; interaction $F_{(9, 99)} = 0.6681$, $P = 0.7358$. Baseline, $P > 0.9999$; 1 day, $P > 0.9999$; 2 days, $P = 0.9996$; 3 days, $P > 0.9999$; 7 days, $P = 0.9235$; 14 days, $P > 0.9999$; 21 days, $P > 0.999$; 28 days, $P = 0.9992$; 35 days, $P > 0.9999$; 42 days, $P > 0.9999$). (**D**) Horizontal ladder test was performed -1, 14, 21, 28, 35, and 42 days after contusion. SCI animals received either 750 μg/kg/hr retigabine or identical vehicle 3 days after contusion for 10 consecutive days. Repeated measures two-way ANOVA followed by Sidak's multiple comparison test (treatment $F_{(1, 10)} = 0.01203$, $p = 0.9148$; time $F_{(5, 50)} = 539$, $P < 0.0001$; interaction $F_{(5, 50)} = 2.862$, $P = 0.0239$. Baseline, $P > 0.999$; 14 days, $P = 0.0856$; 21 days, $P = 0.9608$; 28 days, $P = 0.9929$; 35 days, $P = 0.9884$; 42 days, $P = 0.9054$). *, $P < 0.05$.

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Figure 4. The effects of delayed application of retigabine (750 μg/kg/hr, starting 3 days after contusion for 10 days) via osmotic pump on development of SCI-induced hyperreflexia behaviors. (**A**) Mechanical sensitivity of hindpaws was tested 28 days after initial traumatic spinal cord injury. Each dot in column represent one animal. P = 0.026, U = 26 for SCI + vehicle groups, Mann-Whitney rank sum test; P = 0.03, $t_{(16)}$ = 2.376 for SCI + Retigabine groups; P = 0.9787, $t_{(8)}$ = 0.0275 for sham groups. Two-tailed unpaired t-test. (**B**) Heat hypersensitivity of hindpaws was measured 28 days after initial traumatic spinal cord injury. Each dot in column represent one animal. P = 0.0853, $t_{(20)}$ = 1.811 for SCI + vehicle groups; P = 0.138, $t_{(16)}$ = 1.561 for SCI + Retigabine groups; P = 0.1848, $t_{(8)}$ = 1.451 for sham groups. Two-tailed unpair t-test. (**C**) CPP tests were performed 35 days after contusion. Each dot in column represent one animal. P = 0.0412, $t_{(9)}$ = 2.381, unpaired t-test (two tailed). *, P < 0.05.

Figure 5. The effects of early retigabine application by osmotic pump (750 μg/kg/hr, starting 3 hrs after contusion for 10 days) on SCI-induced activation of glial cells in the L4/L5 spinal cords. (**A**) The effect of treatments on SCI-induced GFAP expression. GFAP was normalized to β-actin. P = 0.0451, $t_{(6)}$ = 2.523, Unpaired t-test (two-tailed). (**B**) The effect of treatments on SCI-induced pSTAT3 expression. pSTAT3 was normalized to β-actin. P = 0.0416, $t_{(6)}$ = 2.583, Unpaired t-test (two-tailed). (**C**) The effect of treatments on SCI-induced Iba-1 expression. Iba-1 was normalized to β-actin. P = 0.9692, $t_{(6)}$ = 0.04028, Unpaired t-test. (**D**) The effect of treatments on SCI-induced expression of phosphorylated MAPK 38. P = 0.9654, $t_{(5)}$ = 0.3787, Unpaired t-test (two-tailed). Dots in each column represents an animal. *, P < 0.05.

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Figure 6. The effects of brief retigabine delivery via osmotic pump (750 μg/kg/hr, starting 3 hrs after contusion for 10 days) on SCI-induced tissue loss in the spinal cord. Representative images in the upper panel showing EC staining of spinal cord sections from both retigabine- and vehicle-treated SCI rats. The graph in the lower panel indicates the quantification of the spared gray matter and white matter of spinal cords at the epicenter of contusion site from vehicle- and retigabine-treated SCI rats. $P = 0.045$, $t_{(5)} = 2.648$ for white matter between vehicle and retigabine groups. Unpaired student t test (two-tailed); $P = 0.229$, $U = 2.0$ for gray matter between vehicle and retigabine groups. Mann-Whitney rank sum test. Dots in each column represents an animal. *, $P < 0.05$. Veh, vehicle; Retig, retigabine.

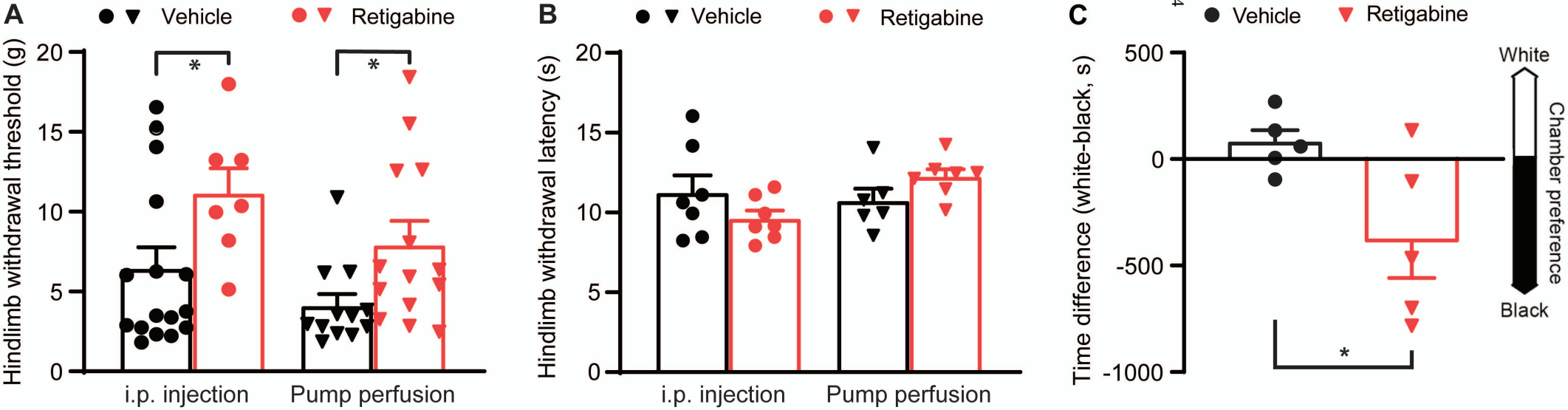
A
Vehicle
Retigabine
Hindlimb withdrawal threshold (g)
20
15
10
5
0
*
*
i.p. injection
Pump perfusion
B
Vehicle
Retigabine
Hindlimb withdrawal latency (s)
20
15
10
5
0
i.p. injection
Pump perfusion
C
Vehicle
Retigabine
Time difference (white-black, s)
500
0
-500
-1000
*
White
Chamber preference
Black

Fig. 1

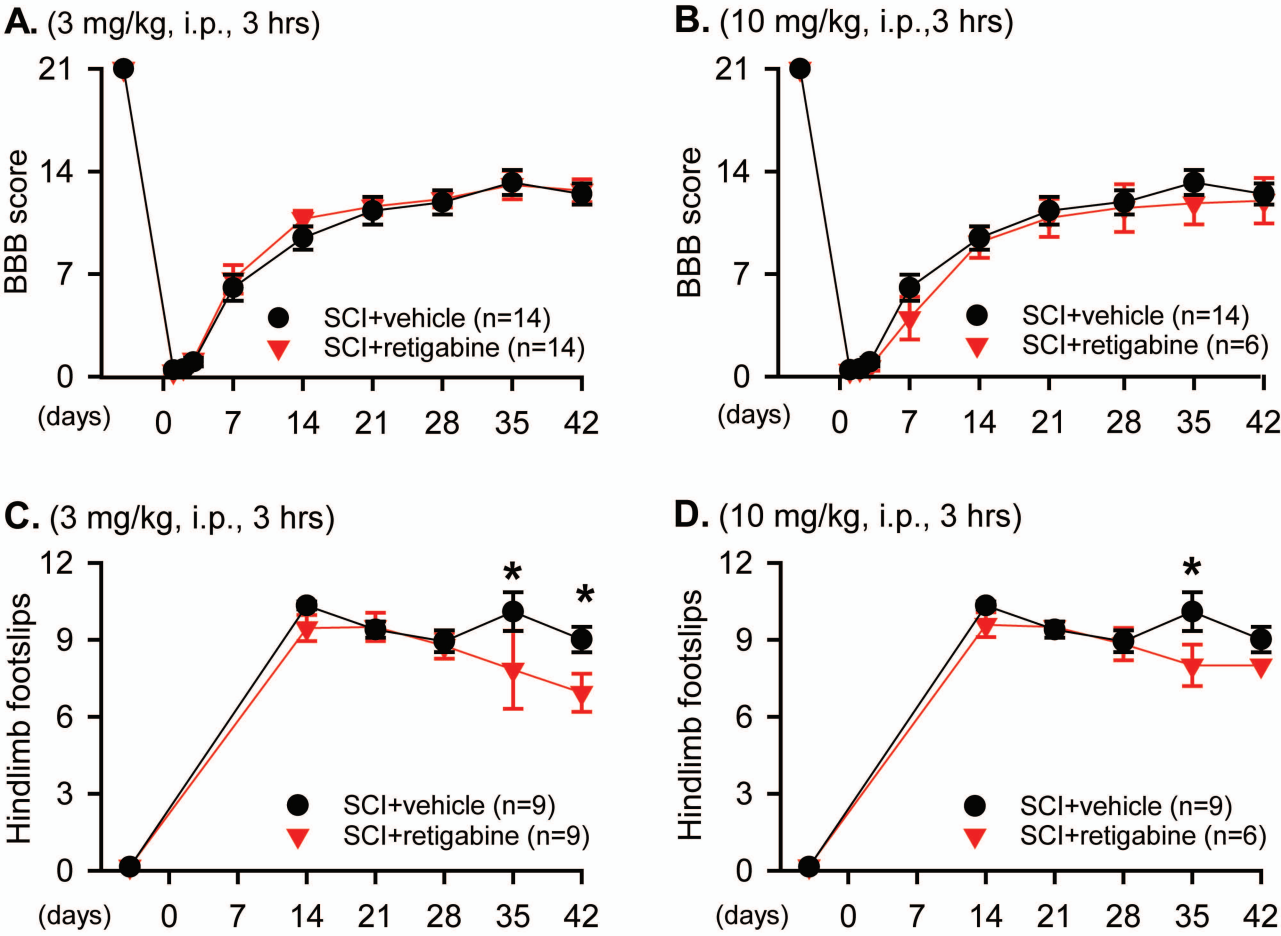
A. (3 mg/kg, i.p., 3 hrs)
B. (10 mg/kg, i.p.,3 hrs)
C. (3 mg/kg, i.p., 3 hrs)
D. (10 mg/kg, i.p., 3 hrs)
BBB score
Hindlimb footslips
(days)
0
7
14
21
28
35
42
SCI+vehicle (n=14)
SCI+retigabine (n=14)
SCI+retigabine (n=6)
SCI+vehicle (n=9)
SCI+retigabine (n=9)
*

Fig. 2

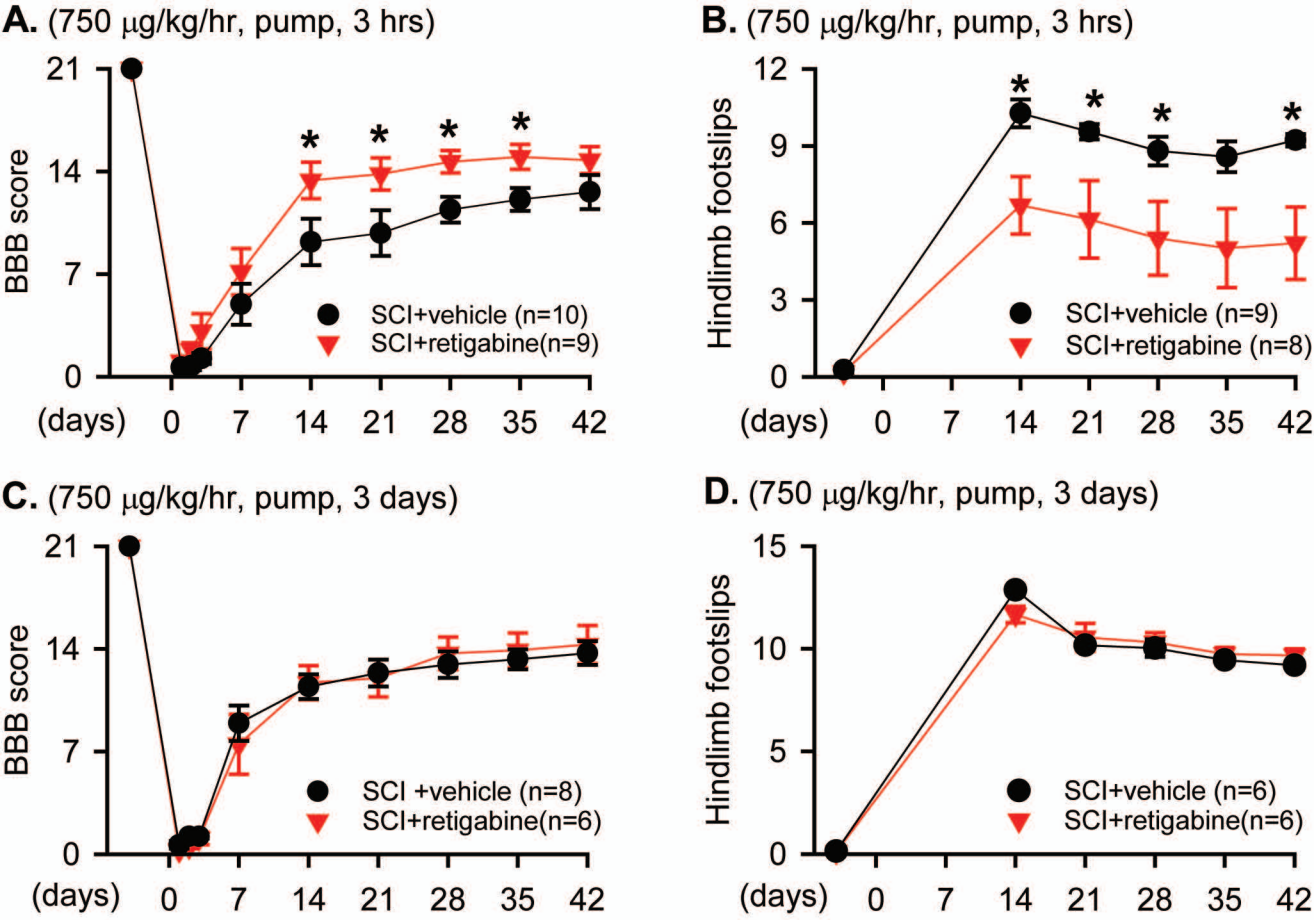
A. (750 μg/kg/hr, pump, 3 hrs)
BBB score
21
14
7
0
(days)
0
7
14
21
28
35
42
*
*
*
*
SCI+vehicle (n=10)
SCI+retigabine(n=9)
B. (750 μg/kg/hr, pump, 3 hrs)
Hindlimb footslips
12
9
6
3
0
(days)
0
7
14
21
28
35
42
*
*
*
*
SCI+vehicle (n=9)
SCI+retigabine (n=8)
C. (750 μg/kg/hr, pump, 3 days)
BBB score
21
14
7
0
(days)
0
7
14
21
28
35
42
SCI +vehicle (n=8)
SCI+retigabine(n=6)
D. (750 μg/kg/hr, pump, 3 days)
Hindlimb footslips
15
10
5
0
(days)
0
7
14
21
28
35
42
SCI+vehicle (n=6)
SCI+retigabine(n=6)

Fig. 3

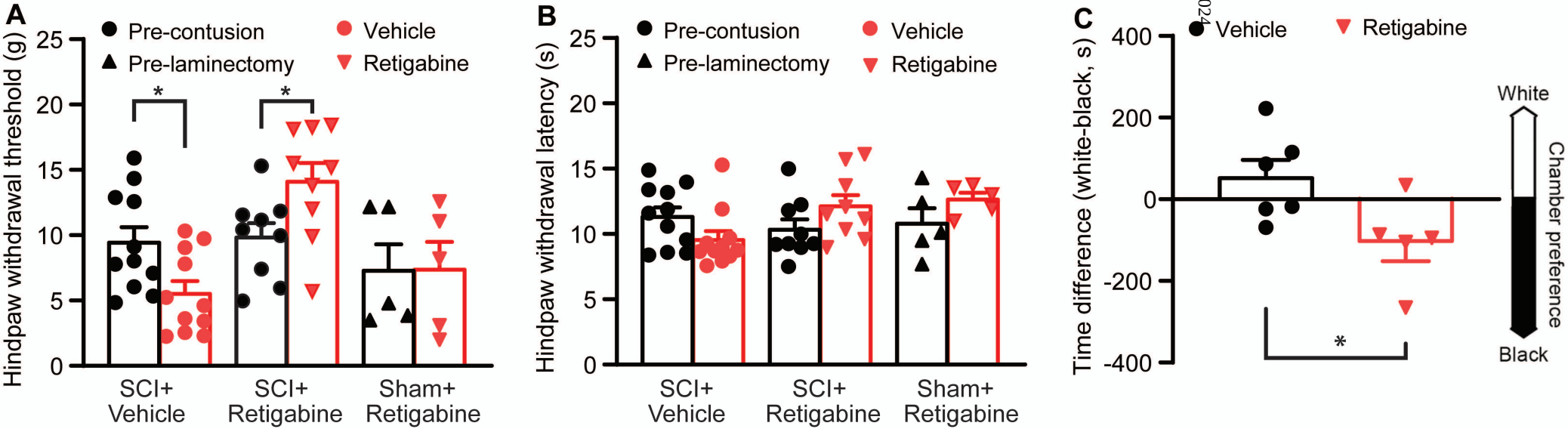
A
Hindpaw withdrawal threshold (g)
Pre-contusion
Pre-laminectomy
Vehicle
Retigabine
*
*
SCI+ Vehicle
SCI+ Retigabine
Sham+ Retigabine
B
Hindpaw withdrawal latency (s)
Pre-contusion
Pre-laminectomy
Vehicle
Retigabine
SCI+ Vehicle
SCI+ Retigabine
Sham+ Retigabine
C
Time difference (white-black, s)
Vehicle
Retigabine
White
Black
Chamber preference
*

Fig. 4

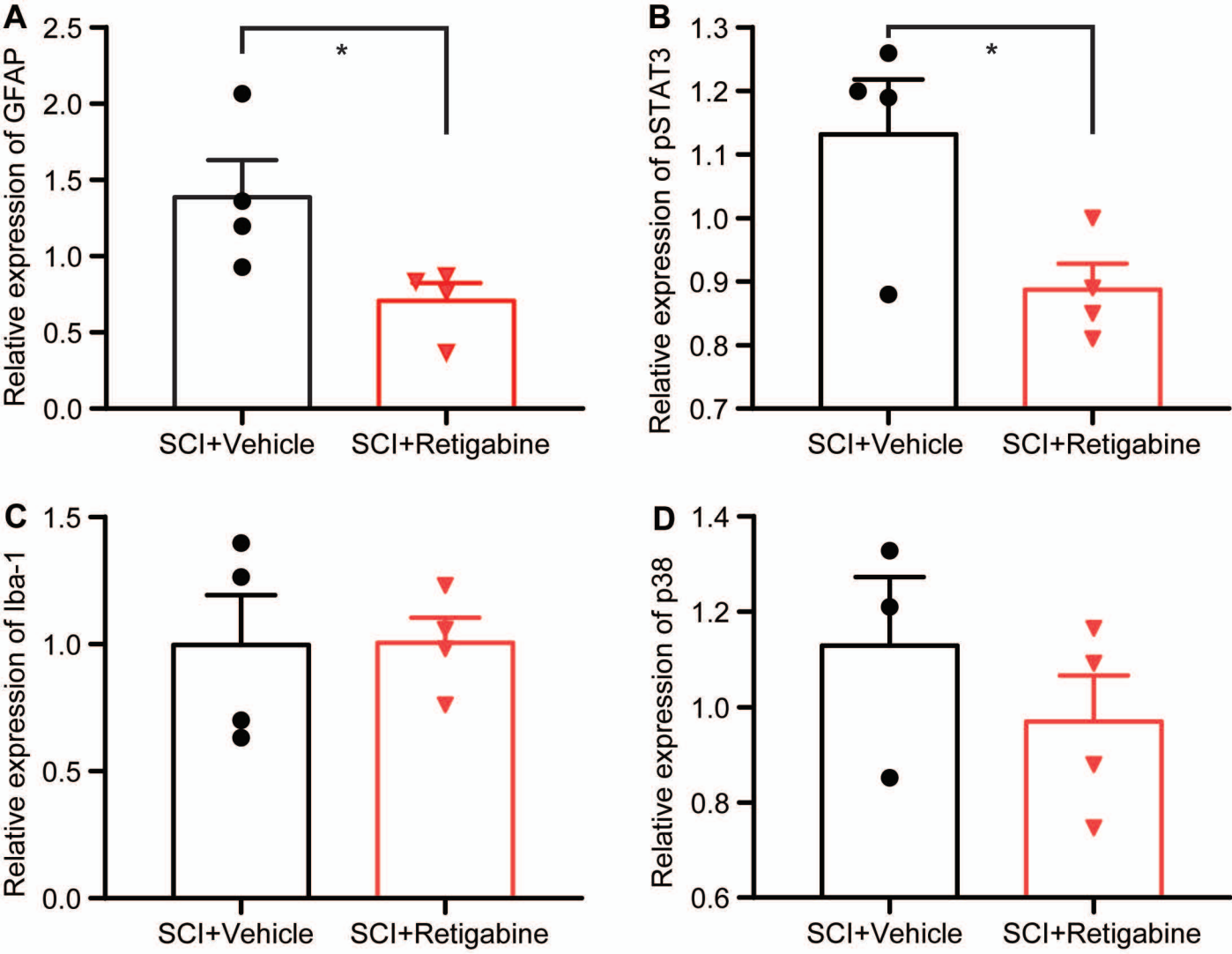
A
Relative expression of GFAP
2.5
2.0
1.5
1.0
0.5
0.0
*
SCI+Vehicle
SCI+Retigabine
B
Relative expression of pSTAT3
1.3
1.2
1.1
1.0
0.9
0.8
0.7
*
SCI+Vehicle
SCI+Retigabine
C
Relative expression of Iba-1
1.5
1.0
0.5
0.0
SCI+Vehicle
SCI+Retigabine
D
Relative expression of p38
1.4
1.2
1.0
0.8
0.6
SCI+Vehicle
SCI+Retigabine

Fig. 5

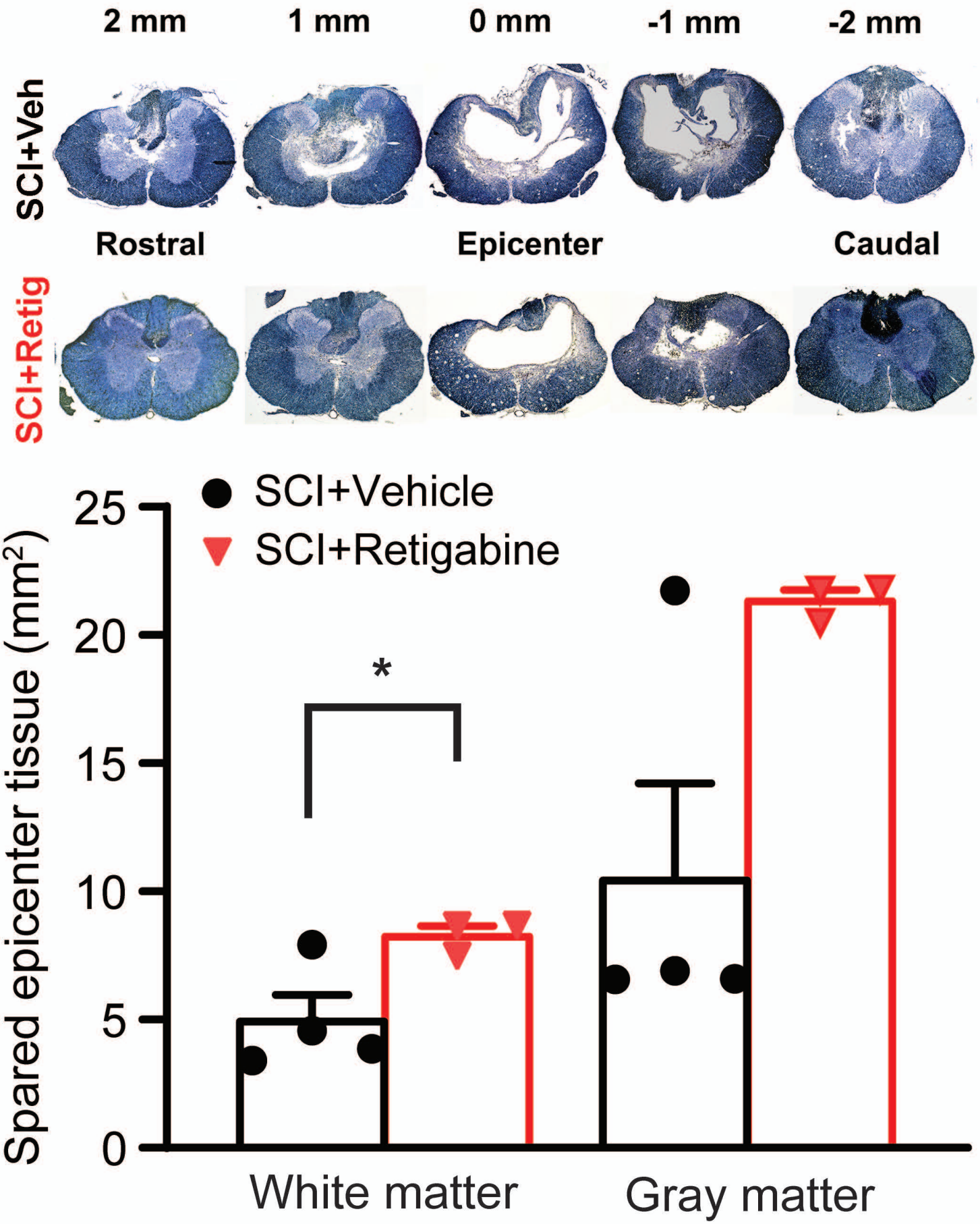
2 mm
1 mm
0 mm
-1 mm
-2 mm
SCI+Veh
Rostral
Epicenter
Caudal
SCI+Retig
SCI+Vehicle
SCI+Retigabine
Spared epicenter tissue (mm²)
25
20
15
10
5
0
*
White matter
Gray matter

Fig. 6