

## **Blocking Proteinase-Activated Receptor 2 Alleviated Neuropathic Pain Evoked by Spinal Cord Injury**

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*Short title:* PAR2 and Neuropathic Pain in Spinal Cord Injury

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## **Summary**

Spinal cord injury (SCI) is an extremely serious type of physical trauma observed in clinics. Especially, neuropathic pain resulting from SCI has a lasting and significant impact on most aspects of daily life. Thus, a better understanding of the molecular pathways responsible for the cause of neuropathic pain observed in SCI is important to develop effectively therapeutic agents and treatment strategies. Proteinase-activated receptors (PARs) are a family member of G-protein-coupled receptors and are activated by a proteolytic mechanism. One of its subtypes PAR2 has been reported to be engaged in mechanical and thermal hyperalgesia. Thus, in this study we specifically examined the underlying mechanisms responsible for SCI evoked-neuropathic pain in a rat model. Overall, we demonstrated that SCI increases PAR2 and its downstream pathways TRPV1 and TRPA1 expression in the superficial dorsal horn of the spinal cord. Also, we showed that blocking spinal PAR2 by intrathecal injection of FSLLRY-NH2 significantly inhibits neuropathic pain responses induced by mechanical and thermal stimulation whereas FSLLRY-NH2 decreases the protein expression of TRPV1 and TRPA1 as well as the levels of substance P and calcitonin gene-related peptide. Results of this study have important implications, i.e., targeting one or more of these signaling molecules involved in activation of PAR2 and TRPV1/TRPA1 evoked by SCI may present new opportunities for treatment and management of neuropathic pain often observed in patients with SCI.

### Key words

Spinal cord injury; Neuropathic pain; Proteinase-activated receptors; TRPV1; TRPA1

## Introduction

Spinal cord injury (SCI) is an extremely serious type of physical trauma observed in medical practice and affects quality of life of more than 2 million people worldwide (Fehlings *et al.* 2012). One of the most common and distressing symptoms suffered by patients with SCI is chronic neuropathic pain. Thus, developing effectively therapeutic agents and treatment strategies for neuropathic pain has clinical importance in patient with SCI. In general, treatment options for these abnormal sensations have been restricted, partly due to a poor understanding of the underlying mechanisms responsible for neuropathic pain induced by SCI.

Notably, SCI leads to obvious changes in the synaptic circuits in the dorsal horn neurons and the areas rostral to the site of injury through a variety of mechanisms (Kumru *et al.* 2010). i.e., there are abnormalities in the expression and activity of receptors and ion channels, release of local inflammatory cytokines and reactive oxygen species, activation of the immune response in microglia and other immune cells, and the activation of intracellular cascades. Nonetheless, the precise mechanisms remain to be decided.

Proteinase-activated receptors (PARs) are a family member of G-protein-coupled receptors and are activated by a proteolytic mechanism (Cottrell *et al.* 2003). Among the four members of PARs, PAR2 is largely distributed in various tissues, including skin, gastrointestinal, cardiovascular, and respiratory systems. Of note, ~60% of dorsal root ganglion (DRG) neurons at the spinal L4-L6 levels contain PAR2 (D'Andrea *et al.* 1998, Steinhoff *et al.* 2000). Stimulation of PAR2 by peripheral or central administration of non-inflammatory doses of PAR2 agonists evokes mechanical and thermal hyperalgesia in rats (Bao *et al.* 2014a, Bao *et al.* 2014b, Bao *et al.* 2015). The studies further suggest that the releases of substance P and calcitonin

gene-related peptide (CGRP) contribute to acute and chronic pain by activation of PAR2. In experimental animal models with neuropathic pain, the expression of PAR2 is upregulated in the dorsal horn of the spinal cord after induction of pain and blocking spinal PAR2 eliminates mechanical and thermal hyperalgesia observed in animals (Chen *et al.* 2011). Nevertheless, it has not been reported that PAR2 pathways specifically contributes to SCI-induced hyperalgesia. The underlying mechanisms responsible for the role of PAR2 in regulating SCI-evoked neuropathic pain are also necessary to be studied.

It has been reported that TRPV1 and TRPA1 are expressed in DRG and as two main receptors they are engaged in neuropathic pain responses (Caterina *et al.* 1997, Jordt *et al.* 2004, Julius 2013, Spicarova *et al.* 2014). In general, the superficial dorsal horn of spinal cord is an important synaptic site to receive pain signal from the peripheral nerves and also has the central neuronal projections to pain-related regions in the central nervous system. A recent study has demonstrated that PAR2 appears in the superficial dorsal horn in involvement of neuropathic pain (Bao *et al.* 2015). Prior findings suggest that PAR2 signaling plays a critical role in regulating TRPV1 and TRPA1 and thereby leading to mechanical allodynia and thermal hyperalgesia (Chen *et al.* 2011, Bao *et al.* 2015). Moreover, PLC, PKA and PKC intracellular signaling pathways are involved on the role of PAR2 (Chen *et al.* 2011, Bao *et al.* 2015).

Therefore, in the present study, we postulated that PAR2 had a regulatory effect on mechanical and thermal hyperalgesia in a rat model of SCI. We hypothesized that SCI increases PAR2 and its downstream pathways TRPV1 and TRPA1 expression in the superficial dorsal horn of the spinal cord. We further hypothesized that blocking spinal PAR2 by intrathecal injection of FSLRY-NH<sub>2</sub> significantly inhibits neuropathic pain responses induced by

mechanical and thermal stimulation whereas FSLLRY-NH2 decreases the protein expression of TRPV1 and TRPA1.

Substance P and CGRP are excitatory neurotransmitters and (or) neuromodulators that are released in the spinal dorsal horn by the primary sensory afferents, thus contributing to the development of allodynia and hyperalgesia by facilitating the release of excitatory glutamate and aspartate from primary afferents (Ma and Eisenach 2003). It should be noted that substance P is restricted to A- and C-fiber nociceptors, the absence of CGRP immunoreactivity in spinal cord may be linked to the absence of alteration of C-fibers (Ma and Eisenach 2003). Thus, we also hypothesized that FSLLRY-NH2 attenuates the amplified levels of substance P and CGRP in the dorsal horn of rats with SCI.

## **Materials and Methods**

### *Animal*

All animal protocols were approved by the Animal Care and Use Committee of our Medical Research Administration and were carried out in accordance with the guidelines of the International Association for the Study of Pain. Male Wistar rats weighing 200-250 g were obtained from the Center for Experimental Animal Sciences. The rats were housed in individual cages with free access to food and water and were kept in a temperature-controlled room (25°C) on a 12/12 h light/dark cycle.

### *A model of spinal cord injury*

There are a number of animal models generally used to study the mechanisms of spinal cord injury. For example, SCI is induced by epidural balloon inflation and by application of

impactor on spinal cord in the rat (Vanicky *et al.* 2001, Urdzikova *et al.* 2006, Hassler *et al.* 2014). In the current study, a total of seventy-eight rats were anesthetized by sodium pentobarbital (40 mg/kg, i.p.) and a laminectomy was then performed to expose spinal segment T10. The Infinite Horizon impactor (150 kdyne, 1 s dwell time) was used to produce contusion spinal injury. Following the injury, the musculature and skin were sutured. The animals were allowed to recover from the surgery. A subcutaneous injection of 0.3 mL of Baytril (25 mg/mL twice daily) was given for 7 days and bladders were manually expressed twice daily. Behavioral test to examine mechanical allodynia and thermal hyperalgesia was performed 4 weeks following SCI.

#### *Intrathecal catheter for administration of drugs*

The rats were anesthetized by sodium pentobarbital (40 mg/kg, i.p.) in order to implant intrathecal catheter for administration of drugs 3 days prior to each experiment. Briefly, one end of polyethylene-10 tubing was inserted intrathecally through an incision in the cisternal membrane and advanced 7-9 cm caudal until the tip of the catheter was positioned at the lumbar spinal level (L5 to L6). The other end of the intrathecal tubing was sutured to the musculature and skin at the incision site and externalized to the back of the rat. In each experiment, a Hamilton microsyringe (250 $\mu$ L) was connected to the intrathecal tubing and used to deliver 100  $\mu$ L of dimethyl sulfoxide (DMSO) as control, FSLLRY-NH<sub>2</sub> (PAR2 antagonist, 10 $\mu$ g), SB366791 (TRPV1 antagonist, 100 $\mu$ M) and HC030031 (TRPA1 antagonist, 10 $\mu$ g) (Kanai *et al.* 2006) (obtained from Sigma-Aldrich).

In a subset of studies, in order to examine the effects of PAR2 on expression of TRPV1 and TRPA1 and engagement of substance P and CGRP FSLLRY-NH<sub>2</sub> (10 $\mu$ g), SB366791

(100 $\mu$ M) and HC030031 (10 $\mu$ g) were intrathecally given using an infusion pump in control rats and rats 4 weeks following SCI, respectively. The pump was set to constantly deliver vehicle or the drugs over a period of 3 hrs. At the end of infusion, the superficial dorsal horn tissues were obtained under an anatomical microscope for Western Blot and ELISA experiments.

### *Behavioral test*

To quantify the mechanical sensitivity of the hindpaw, rats were placed in individual plastic boxes and allowed to acclimate for > 30 min. Mechanical paw withdrawal threshold (PWT) of rat hind paw in response to the stimulation of von Frey filaments was determined. A series of calibrated von Frey filaments (ranging from 0.5 to 18.0 g) were applied perpendicularly to the plantar surface of the hind paw with a sufficient force to bend the filaments for 60s or until paw withdrew. In the presence of a response, the filament of next lower force was applied. In the absence of a response, the filament of next greater force was applied. To avoid injury during tests, the cutoff strength of the von Frey filament was 18 g. The tactile stimulus producing a 50% likelihood of withdrawal was determined using the “up-down” method (Chaplan *et al.* 1994). Each trial was repeated 2 times at approximately 2 min intervals. The mean value was used as the force produced a withdrawal response.

To determine thermal hyperalgesia, rat paw withdrawal latency (PWL) to a radiant heat was measured as described previously (Bao *et al.* 2014a). Rats were placed individually in plastic cages on an elevated glass platform and allowed for 30 min acclimation. Each hind paw received three stimuli with a 10 min interval, and the mean of the three withdrawal latencies was defined as PWL. The heat was maintained at a constant intensity. To prevent tissue damage, the cut-off latency was set at 20 s. All the behavioral tests were performed in a blind style.

### *Western blot analysis*

To examine the protein expression of PAR2, TRPV1 and TRPA1, the superficial dorsal horn tissues were processed using a standard Western Blot procedure (Bao *et al.* 2015). Briefly, the superficial dorsal horn tissues (L4-L6) were removed under an anatomical microscope and total protein was then extracted by homogenizing dorsal horn sample in ice-cold immunoprecipitation assay buffer. The lysates were centrifuged and the supernatants were collected for measurements of protein concentrations. After being denatured by heating at 95°C for 5 min in buffer, the supernatant samples containing 20 µg of protein were loaded onto 4-20% Mini-PROTEAN TGX gels and electrically transferred to a polyvinylidene fluoride membrane. The membrane was blocked in 5% nonfat milk in 0.1% Tween-TBS buffer and was incubated overnight with primary antibody (mouse anti-PAR2, anti-TRPV1 and anti-TRPA1 at 1:200, Cayman Chemical Co.). Next, the membranes were washed and incubated with an alkaline phosphatase conjugated anti-mouse secondary antibody (1:1000). The immunoreactive proteins were detected by enhanced chemiluminescence. The bands recognized by the primary antibody were visualized by exposure of the membrane onto an x-ray film. The membrane was stripped and incubated with mouse anti-β-actin to show equal loading of the protein. Then, the film was scanned and the optical density of PAR2/TRPV1/TRPA1 and β-actin bands was analyzed using the Scion Image software (obtained from the US National Institute of Health).

### *ELISA measurements*

To examine the levels of substance P and CGRP in the superficial dorsal horn of the spinal cord (L4-L6), ELISA methods were employed. Substance P was measured using substance P ELISA kit following the manufacturer's instructions (Abcam Co.). Briefly, the



diluted tissue supernatant (100  $\mu$ l) was placed in a 96-well goat anti-mouse IgG-coated plate and incubated for 2 hours. After incubation, the plate was washed using the provided washing buffer, and the color was developed by adding PNPP (200  $\mu$ l) substrate after 45 min and determined by an ELISA plate reader. The amount of substance P was calculated by using a substance P standard curve. In the similar way, the CGRP content of the samples (100  $\mu$ l supernatant) was determined using a commercial CGRP ELISA kit (Cayman Chemical Co.). Briefly, the diluted samples were placed in a 96-well plate incubated with pre-coated anti-rat IgG antibody overnight, washed and developed, and quantified (Wang *et al.* 2014).

### *Statistical analysis*

All data were analyzed using a one-way analysis of variance. Values were presented as means  $\pm$  standard error of mean (SEM). For all analyses, differences were considered significant at  $P < 0.05$ . All statistical analyses were performed by using SPSS for Windows version 13.0 (SPSS).

## **Results**

In order to obtain baseline values, responses to the mechanical and thermal stimulation were examined prior to SCI surgery (n=78). Basal PWT was  $14.4 \pm 1.2$  g and basal PWL was  $12.5 \pm 1.1$  s. Mechanical allodynia and thermal hyperalgesia began to appear 2 weeks after induction of SCI and lasted for 4 weeks. In those rats, PWT was  $5.5 \pm 0.6$  g ( $P < 0.05$  vs. baseline) and PWL was  $5.2 \pm 0.5$  s ( $P < 0.05$  vs. baseline) 4 weeks following SCI. Note that no behavioral test was performed  $>4$  weeks in this experiment. Five out of 83 animals (~6%) that did not display increases in mechanical and thermal sensitivity of at least 40% of baseline values were excluded from the experiment.

### *PAR2 engaged in SCI-induced hyperalgesia*

Figure 1 (left panel) showed the protein expression of PAR2 in control rats and rats with SCI. SCI significantly increased the protein levels of PAR2 in the superficial dorsal horn of the spinal cord as compared with control rats (\* $P < 0.05$ , SCI rats vs. control rats,  $n = 8$  in each group).

In another group of experiments (middle and right panels), after mechanical and thermal hyperalgesia were well established rats were treated with intrathecal injection of FSLLRY-NH2 (10 $\mu$ g) (Bao *et al.* 2014a) and mechanical and thermal sensitivities were examined at 0, 1, 2, 3, 4, 5, 6, 7 and 8 hrs after injection. Figure 1 showed that FSLLRY-NH2 ( $n = 16$ ) significantly attenuated SCI-induced mechanical and thermal hyperalgesia ( $P < 0.05$  vs. vehicle control,  $n = 12$ ). The inhibitory effects of FSLLRY-NH2 on mechanical and thermal hyperalgesia began from ~60 min after its administration, peaked at 2-3 hrs and lasted for 6 hrs.

### *TRPV1 and TRPA1 pathways in mediating SCI-induced hyperalgesia*

We also examined the effects of TRPV1 and TRPA1 pathways on PAR2-mediated hyperalgesia in SCI rats. Figure 2 (left panel) demonstrated that expression of TRPV1 was significantly increased in SCI rats ( $n = 10$ ) as compared with control rats ( $n = 10$ ). In another group, FSLLRY-NH2 was infused into the spinal cord of SCI rats and the expression of TRPV1 activities was examined. We found that FSLLRY-NH2 significantly attenuated the amplified TRPV1 activities in the dorsal horn of SCI rats ( $n = 12$ ).

In addition, in this study, SB366791 (100 $\mu$ M, a TRPV1 inhibitor) (Uchytlova *et al.* 2014) was intrathecally injected. Figure 2 (middle and right panels) demonstrated that SB366791 had

significant attenuating effects on SCI-induced mechanical and thermal hyperalgesia in a time manner (\* $P < 0.05$ , SB366791 vs. vehicle,  $n = 12$  for each group). At this dose, the effects of SB366791 appeared at ~60 min, peaked at 2-3 hrs and lasted for ~6 hrs after injection.

Likewise, Figure 3 (left panel) showed that expression of TRPA1 was significantly increased in SCI rats ( $n = 10$ ) as compared with control rats ( $n = 10$ ). FSLLRY-NH2 infused into the spinal cord of SCI rats significantly attenuated the amplified TRPA1 expression in the dorsal horn of SCI rats ( $n = 12$ ). Figure 3 (middle and right panels) further showed that intrathecal injection of HC030031 (10 $\mu$ g,  $n = 14$ ) led to a time-dependent inhibitory effect on SCI-induced mechanical and thermal hyperalgesia (\* $P < 0.05$ , HC030031 vs. vehicle,  $n = 12$ ). At this dose, the effects of HC030031 appeared at ~60 min, peaked at 2-3 hrs and lasted for ~5 hrs after injection.

#### *The levels of substance P and CGRP*

In additional experiments, the effects of SCI on the levels of substance P and CGRP in the superficial dorsal horn of the spinal cord were examined as shown in Figure 4. Substance P and CGRP were significantly increased in SCI rats ( $n = 16$ ) as compared with control rats ( $n = 12$ ). Furthermore, blocking individual PAR2, TRPV1 and TRPA1 by intrathecal injection of 10 $\mu$ g of FSLLRY-NH2 ( $n = 10$ ), 100 $\mu$ M of SB366791 ( $n = 12$ ) and 10 $\mu$ g of HC030031 ( $n = 12$ ) significantly attenuated amplifications in substance P and CGRP evoked by SCI. Note that a greater inhibitory effect on substance P and CGRP was observed by FSLLRY-NH2 compared with SB366791 or HC030031 given alone.

## **Discussion**

Data of the current study demonstrated that expression of PAR2 in the superficial dorsal horn of SCI rats was upregulated, and PAR2 antagonist FSLLRY-NH2 attenuated mechanical and thermal hyperalgesia evoked by SCI. FSLLRY-NH2 also attenuated heightened protein expression of TRPV1 and TRPA1 receptors engaged in mechanical and thermal hypersensitivity evoked by SCI. In addition, the levels of substance P and CGRP, two important neurotransmitters engaged in the neuropathic pain, were significantly increased in the superficial dorsal horn of SCI rats and intrathecal injection of respective FSLLRY-NH2, SB366791 and HC030031 significantly attenuated the increased substance P and CGRP. Notably, FSLLRY-NH2 had a greater effect on substance P and CGRP than either SB366791 or HC030031 did. Thus, our data suggest that amplified expression of spinal PAR2 and its downstream pathways TRPV1 and TRPA1 are likely engaged in SCI-induced mechanical and thermal hyperalgesia via the releases of substance P and CGRP.

TRPV1 is a nonselective cation channel that can be activated by a wide variety of endogenous physical and chemical stimuli such as noxious heat, low pH (acidic conditions), endocannabinoid anandamide, N-oleyl-dopamine, and N-arachidonoyl-dopamine etc (Caterina *et al.* 1997, Caterina *et al.* 2000, Davis *et al.* 2000). The activation of TRPV1 leads to a painful, burning sensation. TRPV1 receptors are found mainly in the nociceptive neurons of the peripheral nervous system, but they have also been described in the central nervous system including brain and spinal cord (Julius 2013, Bevan *et al.* 2014, Peppin and Pappagallo 2014, Spicarova *et al.* 2014). TRPV1 is involved in the transmission and modulation of pain (nociception), as well as the integration of diverse painful stimuli (Julius 2013, Bevan *et al.* 2014, Peppin and Pappagallo 2014, Spicarova *et al.* 2014). Evidence further suggests the role for TRPV1 in regulating neuropathic pain in peripheral and central nervous systems (Peppin and

Pappagallo 2014). Results of our current study support the specific role played by TRPV1 at the level of spinal cord in regulating mechanical and thermal hyperalgesia evoked by SCI.

Moreover, PAR2 plays a role in regulating spinal TRPV1 expression and its engagement of SCI-evoked neuropathic pain.

Also, TRPA1 has a functional role in pain and neurogenic inflammation resulting from channel activation to a variety of compounds including pungent agents, irritant chemicals, reactive oxygen and nitrogen species, and products of oxidative stress-induced lipid peroxidation (Bandell *et al.* 2004, Jordt *et al.* 2004, Bautista *et al.* 2005, Andersson *et al.* 2008, Sawada *et al.* 2008). TRPA1 has been shown to co-localize with TRPV1 in subpopulations of DRG neurons (Jordt *et al.* 2004) and is engaged in development of bradykinin-induced mechanical hypersensitivity and (Story *et al.* 2003, Kwan *et al.* 2006) painfully cold temperatures (Zhao *et al.* 2012). Our evidence from the current study supports the notion that TRPA1 mediates SCI-induced mechanical and thermal hypersensitivity. Results of our current study further suggest that PAR2 plays an important role in regulating TRPA1 functions in SCI-evoked neuropathic pain because blocking PAR2 significantly attenuates the protein expression of TRPA1 in the dorsal horn engaged in SCI-evoked mechanical and thermal hyperalgesia.

In the current study, we identified a greater level of PAR2 expression in the superficial dorsal horn of spinal cord of rats with neuropathic pain following SCI. This is consistent with findings in a prior study showing upregulation of PAR2 in the superficial dorsal horn of a rat model with cancer-induced neuropathic pain (Bao *et al.* 2015). TRPV1 and TRPA1 have been reported to regulate neuropathic pain responses at spinal cord level (Caterina *et al.* 1997, Jordt *et al.* 2004, Julius 2013, Spicarova *et al.* 2014). Additional results suggest that PAR2 signaling plays a critical role in regulating TRPV1 and TRPA1 and thereby leading to mechanical allodynia

and thermal hyperalgesia (Chen *et al.* 2011, Bao *et al.* 2015). Moreover, PLC, PKA and PKC intracellular signaling pathways are involved on the role of PAR2 (Chen *et al.* 2011, Bao *et al.* 2015). It was observed that SCI amplifies expression of PAR2 in the superficial dorsal horn in our current study and speculatively this is likely to activate PLC, PKA and PKC signaling pathways and then lead to increases of TRPV1 and TRPA1 in regulating mechanical allodynia and thermal hyperalgesia in a model of SCI.

It is well reasoned that SCI increases the amount of substance P and CGRP observed in our current study likely via stimulation of A-fibers and/or C-fibers. Stimulation of TRPV1 or TRPA1 in the dorsal horn alters the releases of substance P and CGRP (Lin *et al.* 2007, Bevan *et al.* 2014). Nevertheless, to the best of our knowledge there is lacking of evidence specifically showing the role played by TRPV1 and TRPA1 in regulating the releases of spinal substance P and CGRP in a neuropathic pain model induced by SCI. Results of the present report suggest that substance P and CGRP regulated by TRPV1 and/or TRPA1 at the spinal level contribute to SCI-induced neuropathic pain.

## **Conclusions**

Inhibition of spinal PAR2 and its downstream TRPV1 and TRPA1 receptors antagonizes mechanical and thermal hyperalgesia following induction of SCI. Protein expression of TRPV1 and TRPA1 receptors are upregulated by SCI, and TRPV1 and TRPA1 pathways play a role in PAR2 regulating SCI-induced neuropathic pain. Results of our study will provide a base for the mechanisms responsible for SCI-induced neuropathic pain and further offer a strategy to target the spinal neuronal levels for treatment and management of neuropathic pain often observed in patients with SCI. In addition, targeting one or more of these signaling molecules involved in

activation of PAR2, TRPV1 and TRPA1 evoked by SCI may present new opportunities for treatment and management of neuropathic pain often observed in SCI patients.

### Conflict of interest

None

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## Figure Legends

**Figure 1. Mechanical and thermal hyperalgesia developed after SCI.** Left panel: Typical bands and averaged data showing that PAR2 in the dorsal horn of the spinal cord was upregulated in SCI rats (n=8). \* $P < 0.05$  vs. control rats (n=8). Middle and right panels: Intrathecal administration of FSLLRY-NH2 increased PWT and PWL in SCI rats. \* $P < 0.05$  vs. DMSO as vehicle control. N=12 in controls and n=16 in group of FSLLRY-NH2.

**Figure 2. Effects of FSLLRY-NH2 on expression of TRPV1 engaged in SCI-induced hyperalgesia.** Left panel: Typical band and averaged data showing that expression of TRPV1 was increased in the dorsal horn of SCI rats (n=10) and FSLLRY-NH2 infused into the spinal cord attenuated an increase of TRPV1. \* $P < 0.05$  vs. control rats (n=10) and SCI rats infused with FSLLRY-NH2 (n=12). Middle and right panels: Showing that intrathecal injection TRPV1 antagonist, SB366791, attenuated mechanical and thermal hyperalgesia in SCI rats (n=12). \* $P < 0.05$  vs. DMSO injection (n=12).

**Figure 3. Effects of FSLLRY-NH2 on expression of TRPA1 engaged in SCI-induced hyperalgesia.** Left panel: Typical band and averaged data showing that expression of TRPA1 was increased in the dorsal horn of SCI rats (n=10) and FSLLRY-NH2 infused into the spinal cord attenuated an increase of TRPV1. \* $P < 0.05$  vs. control rats (n=10) and SCI rats infused with FSLLRY-NH2 (n=12). Middle and right panels: Showing that intrathecal injection TRPA1 antagonist, HC030031, attenuated mechanical and thermal hyperalgesia in SCI rats (n=14). \* $P < 0.05$  vs. DMSO injection (n=12).

**Figure 4. The levels of substance P and CGRP in the superficial dorsal horn of the spinal cord.** SCI significantly increased substance P and CGRP as compared with controls and blocking PAR2, TRPV1 and TRPA1 by intrathecal injection FSLLRY-NH2 (10 $\mu$ g), SB366791

(100 $\mu$ M) and HC030031 (10 $\mu$ g) significantly attenuated the enhancement in substance P and CGRP evoked by SCI. Note that a greater inhibitory effect on substance P and CGRP was observed by FSLLRY-NH<sub>2</sub>. Data are expressed as mean  $\pm$  SEM. \* $P$  < 0.05, indicated SCI-rats (n=16) vs. control rats (n=12) and SCI-rats with FSLLRY-NH<sub>2</sub> (n=10), SB366791 (n=12) and HC030031 injection (n=12). #  $P$  < 0.05, indicated SCI rats with FSLLRY-NH<sub>2</sub> injection vs. with respective SB366791 and HC030031 injection.

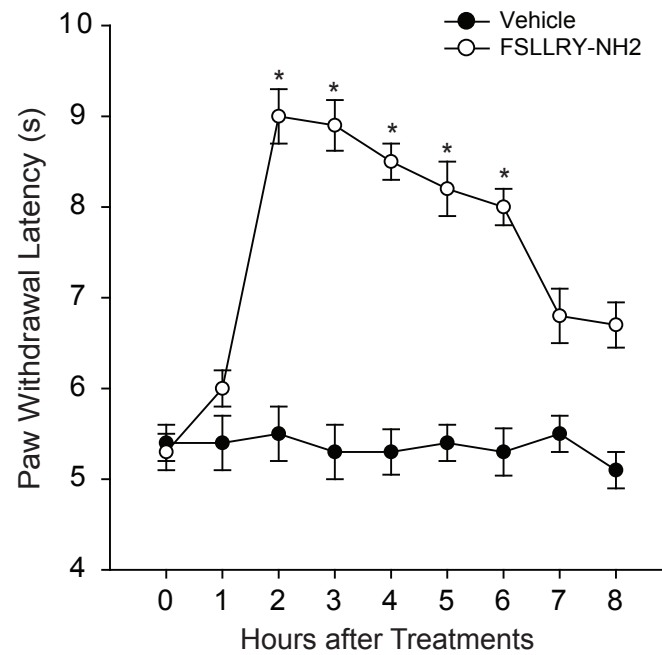
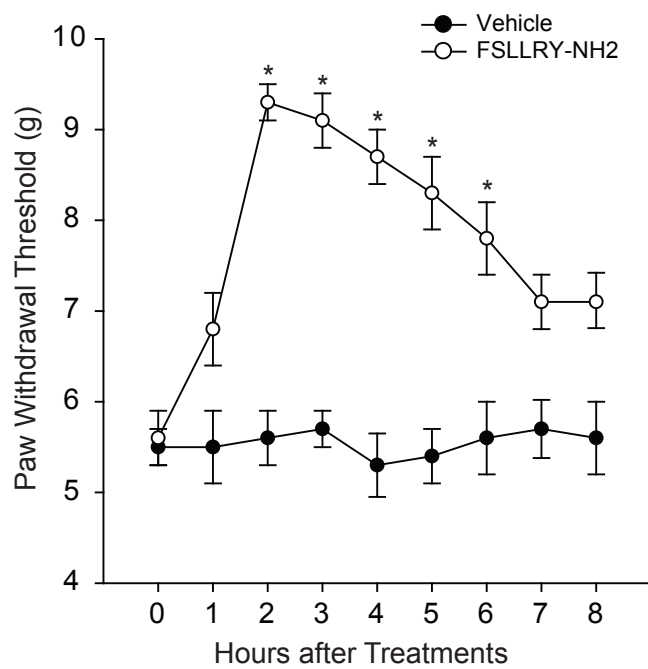
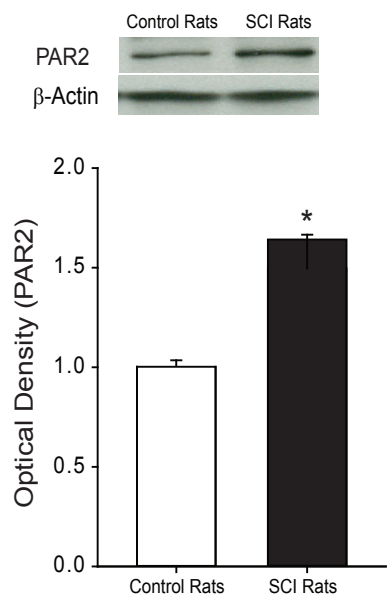


Figure 1

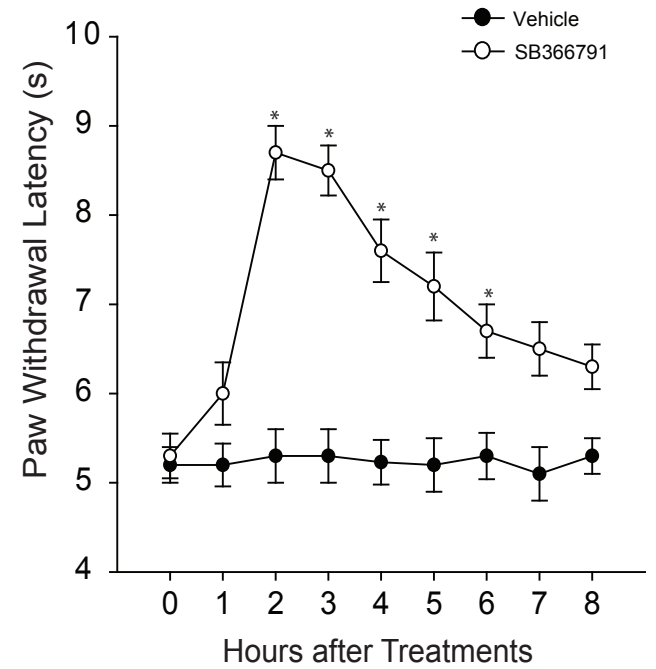
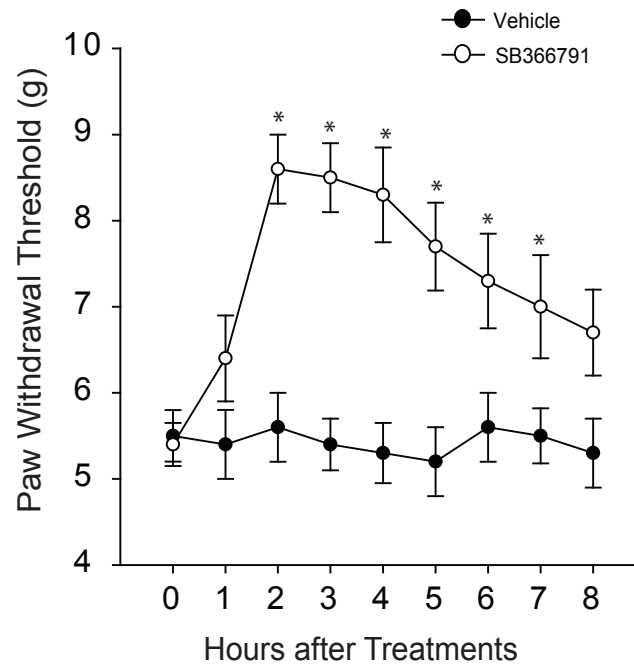
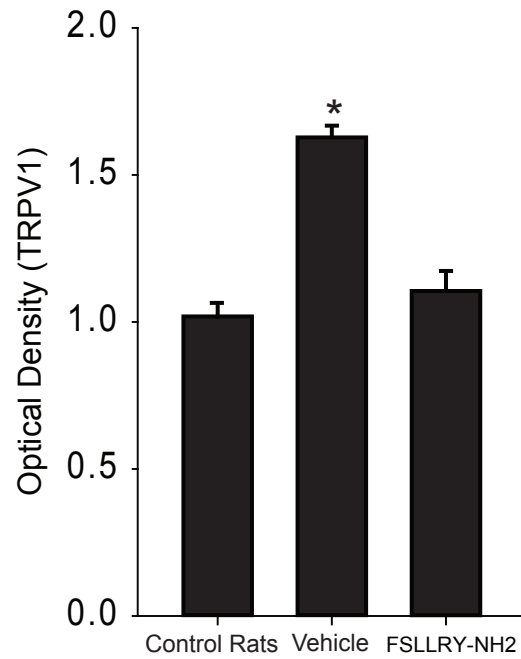
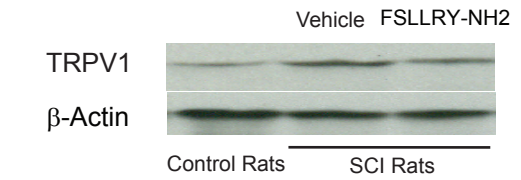


Figure 2

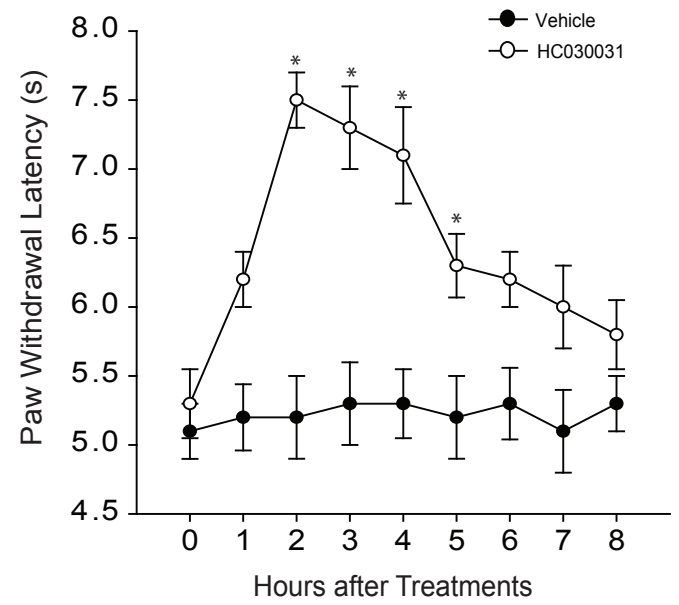
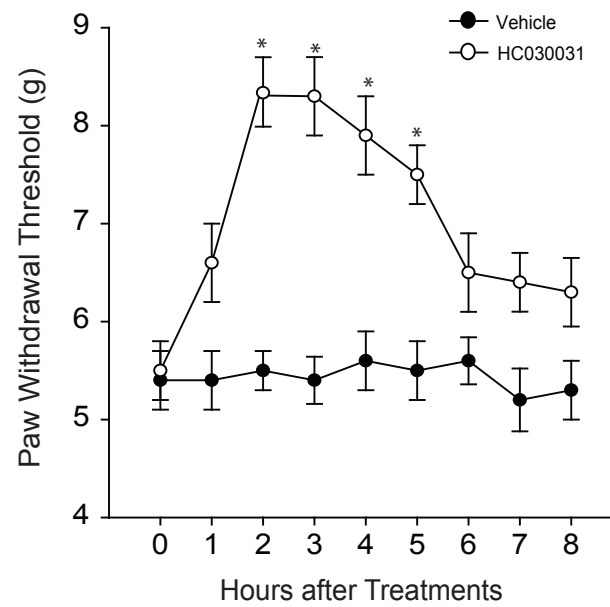
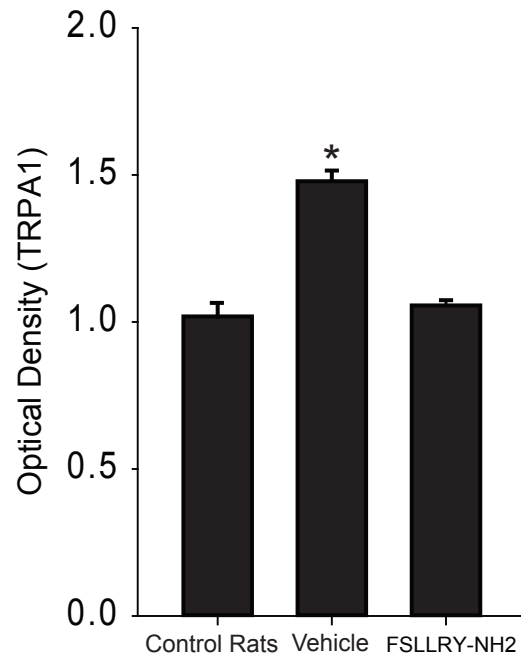
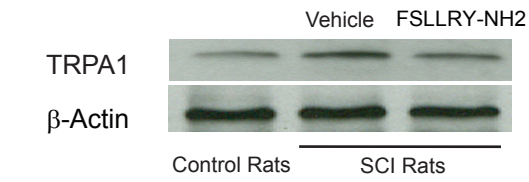


Figure 3

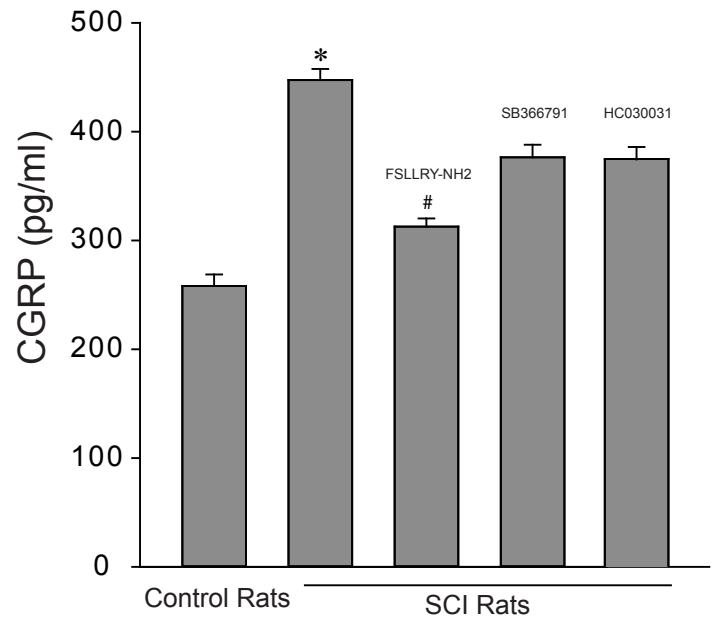
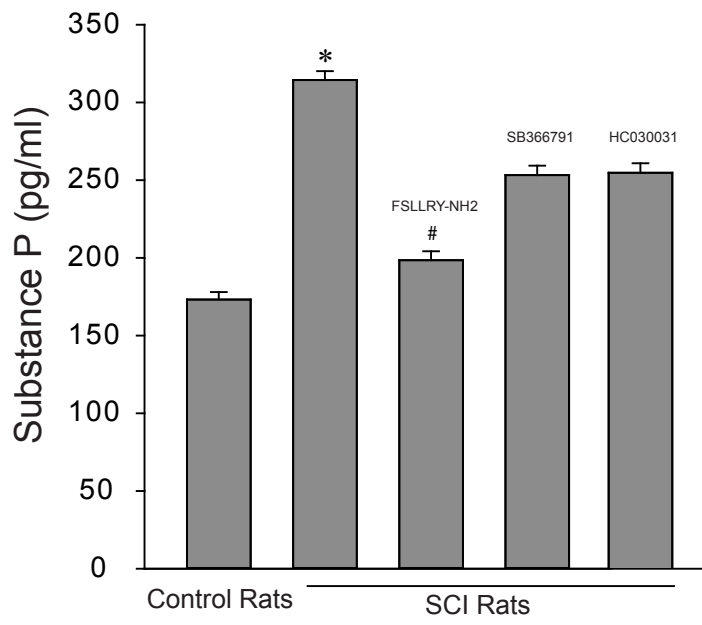


Figure 4