

## DESCENDING FACILITATION FROM THE ROSTRAL VENTROMEDIAL MEDULLA MAINTAINS NERVE INJURY-INDUCED CENTRAL SENSITIZATION

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**Abstract**—Nerve injury can produce hypersensitivity to noxious and normally innocuous stimulation. Injury-induced central (i.e. spinal) sensitization is thought to arise from enhanced afferent input to the spinal cord and to be critical for expression of behavioral hypersensitivity. Descending facilitatory influences from the rostral ventromedial medulla have been suggested to also be critical for the maintenance, though not the initiation, of experimental neuropathic pain. The possibility that descending facilitation from the rostral ventromedial medulla is required for the maintenance of central sensitization was examined by determining whether ablation of mu-opioid receptor-expressing cells within the rostral ventromedial medulla prevented the enhanced expression of repetitive touch-evoked FOS within the spinal cord of animals with spinal nerve ligation injury as well as nerve injury-induced behavioral hypersensitivity. Rats received a single microinjection of vehicle, saporin, dermorphin or dermorphin–saporin into the rostral ventromedial medulla and 28 days later, underwent either sham or spinal nerve ligation procedures. Animals receiving rostral ventromedial medulla pretreatment with vehicle, dermorphin or saporin that were subjected to spinal nerve ligation demonstrated both thermal and tactile hypersensitivity, and showed significantly increased expression of touch-evoked FOS in the dorsal horn ipsilateral to nerve injury compared with sham-operated controls at days 3, 5 or 10 post-spinal nerve ligation. In contrast, nerve-injured animals pretreated with dermorphin–saporin showed enhanced behaviors and touch-evoked FOS expression in the spinal dorsal horn at day 3, but not days 5 and 10, post-spinal nerve ligation when compared with sham-operated controls. These results indicate the presence of nerve injury-induced behavioral hypersensitivity associated with nerve injury-induced central sensitization. Further, the results demonstrate the novel concept that once initiated, maintenance of nerve injury-induced central sensitization in the spinal dorsal horn requires descending pain facilitation mechanisms arising from the rostral ventromedial medulla. © 2006 IBRO. Published by Elsevier Ltd. All rights reserved.

**Key words:** neurochemical injury, non-noxious stimulation, immunohistochemistry.

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**Abbreviations:** CFA, complete Freund's adjuvant; DERM-SAP, dermorphin–saporin; DLF, dorsolateral funiculus; LC/SC, locus coeruleus/subcoeruleus; NGC, nucleus gigantocellularis; NGS, normal goat serum; NRM, nucleus raphe magnus; PBS, phosphate buffer solution; RVM, rostral ventromedial medulla; SNL, spinal nerve ligation.

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Peripheral nerve injury can result in neuropathic pain characterized clinically by spontaneous burning pain, allodynia to touch and cold and hyperalgesia to noxious stimuli (Payne, 1986; Merskey and Bogduk, 1994; White, 2004). Clinical studies performed with selective nerve blocks along with electrophysiology studies performed in animals have led to the generally accepted conclusion that enhanced pain induced by peripheral nerve injury is associated with increased spontaneous and evoked discharges from injured and/or adjacent uninjured nerves (Koltzenburg et al., 1992, 1994 Amir and Devor, 2000; Michaelis et al., 2000; Liu et al., 2001). Persistent primary afferent inputs are believed to cause a state of central (i.e. "spinal") sensitization, enhancing responses to sensory inputs and thus maintaining an enhanced pain state in a manner with some similarities to long-term potentiation (Ren, 1994; Chapman et al., 1998; Ziegler et al., 1999; Suzuki and Dickenson, 2000; Dickenson et al., 2001).

While increased afferent discharge is critical in establishing the process of spinal sensitization in the period immediately following nerve injury, the time-course of such abnormal afferent activity is inconsistent with the long duration of enhanced pain (Chaplan et al., 1994; Malan et al., 2000; Porreca et al., 2002; Burgess et al., 2002). It was therefore suggested that additional mechanisms may also play an essential role in the maintenance of the neuropathic state. Extensive behavioral and electrophysiological studies have demonstrated that bulbospinal facilitatory projections from the rostral ventromedial medulla (RVM) can promote tactile and thermal hyperesthesias (Morgan and Fields, 1994; Urban et al., 1996; Zhuo and Gebhart, 1997; Urban and Gebhart, 1999). Recent evidence has established that the RVM is critical to the maintenance, although not to the initiation, of behavioral signs of neuropathic pain (Porreca et al., 2001; Burgess et al., 2002). Microinjection of lidocaine into the RVM abolished behavioral signs of allodynia and hyperalgesia in nerve-injured rats (Pertovaara et al., 1996; Kovelowski et al., 2000; Burgess et al., 2002). Similarly, surgical disruption of descending RVM projections through lesioning the dorsolateral funiculus (DLF) or the selective ablation with dermorphin–saporin conjugate of RVM neurons that express the mu-opioid receptor, presumed to be a source of spinopetal facilitatory projections, also abolished behavioral signs of neuropathic pain (Ossipov et al., 2000; Porreca et al., 2001; Burgess et al., 2002). Importantly, in these studies, the reversal of the behavioral endpoints was only evident 6 days or more after spinal nerve ligation (SNL), suggesting

that other mechanisms may contribute to the early phase of neuropathic pain state.

The expression of the proto-oncogene product FOS is a widely accepted marker indicative of transynaptic excitation of spinal dorsal horn neurons, and is associated with noxious stimuli (Hunt et al., 1987; Harris, 1998). While repetitive light tactile stimuli do not normally elicit significant FOS expression in the spinal dorsal horn, such stimuli do produce significant FOS expression in conditions of central sensitization, such as in experimental nerve injury (Hunt et al., 1987; Presley et al., 1990; Molander et al., 1998; Shortland and Molander, 1998; Catheline et al., 1999, 2001). In the present study, repetitive touch-evoked FOS was utilized as an indicator of central sensitization in rats with peripheral nerve injury along with behavioral measures of enhanced pain in order to determine a possible role of descending facilitation in the initiation and maintenance of injury-induced central sensitization.

## EXPERIMENTAL PROCEDURES

Male, Sprague–Dawley rats (Harlan, Indianapolis, IN, USA), weighing 200–300 g at the time of surgery were maintained in a climate-controlled room on a 12-h light/dark cycle (light on at 07:00 h). Food and water were available *ad libitum*. All testing and surgeries were performed in accordance with the policies and recommendations of the International Association for the Study of Pain and the National Institutes of Health guidelines for the handling and use of laboratory animals. These studies also received approval from the Institutional Animal Care and Use Committee of the University of Arizona. All efforts were made to minimize the number of animals used and their suffering.

### Intracranial drug microinjection

All animals were prepared for bilateral microinjections into the RVM as described previously (Burgess et al., 2002). The rats were anesthetized with 100 mg/kg of ketamine and 10 mg/kg of xylazine and positioned in a stereotaxic head holder. The skull was exposed and two 26 ga guide cannulas separated by 1.2 mm (Plastics One Inc., Roanoke, VA, USA) were directed toward the lateral portions of the RVM (anteroposterior, –11.0 mm from Bregma; lateral,  $\pm 0.6$  mm from midline; dorsoventral –8.5 mm from the cranium) (Paxinos and Watson, 1986). The guide cannulae were secured to the skull, and the animals were allowed to recover for 5 days after surgery before any drug administration. All surgeries were done by the same experimenter (J.Y.X.). Drug administration into the RVM was performed by slowly expelling 0.5  $\mu$ l of drug solution through a 33 ga injection cannula inserted through the guide cannula and protruding an additional 1 mm into fresh brain tissue to prevent backflow of the drug into the injection cannula. Dermorphin, saporin and dermorphin–saporin (Advanced Targeting Systems, San Diego, CA, USA), or vehicle (water) was administered as a single dose of 3 pmol into the RVM (1.5 pmol in 0.5  $\mu$ l each side). The cannulae were left in place to be able to confirm the microinjection sites at the end of the experiments in the same animals. As dermorphin, and dermorphin–saporin show better solubility in distilled water than in saline, we used distilled water as the vehicle control. Distilled water did not have any discernible effect either at the time of injection or at the 28 day time point or thereafter.

### SNL

Twenty-eight days after RVM microinjections, the rats were prepared with SNL or sham surgery as described by Kim and Chung

(1992). Anesthesia was induced with 2% halothane in O<sub>2</sub> at a rate of 2 l/min and maintained with 0.5% halothane in O<sub>2</sub>. After surgical preparation of the rats and exposure of the dorsal vertebral column from L<sub>4</sub> to S<sub>2</sub>, the L<sub>5</sub> and L<sub>6</sub> spinal nerves were tightly ligated distal to the DRG with a 4-0 silk suture. The incision was closed and the animals were allowed to recover for 3 days. Rats that exhibited motor deficiency or failure to exhibit mechanical and/or thermal hypersensitivity (less than 10%) were excluded from further testing. Sham control rats underwent the same operation and handling as the experimental animals, but the spinal nerves were not ligated.

### Behavioral testing

Mechanical and thermal testing took place before SNL surgery and then 3, 5 and 10 days after SNL or sham surgery. Responses to tactile stimuli were determined by the method described by (Chaplan et al., 1994). The rats were acclimated in suspended Plexiglas cages with wire mesh floors. The hindpaws were probed with a series of eight von Frey filaments (Stoelting, Wood Dale, IL, USA) with ascending, logarithmically spaced increments of stiffness ranging from 0.41 g to 15 g (4 mN to 150 mN). Each filament was applied perpendicularly to the plantar surface of the ligated hindpaw until a withdrawal response occurred for 6 s. The probes were applied in an ascending/descending fashion until three transitions between response and non-response occurred. The pattern of response was analyzed by the Dixon non-parametric test in order to estimate the paw withdrawal threshold (Chaplan et al., 1994). A significant ( $P < 0.05$ ) decrease in response threshold from the pre-SNL baseline level indicated tactile hyperesthesia.

Responses to thermal stimuli were determined according to the method described by Hargreaves et al. (1988). Rats were acclimated to Plexiglas cages placed over a glass plate. An infrared heat source was projected onto the plantar aspect of the hindpaw, and the paw withdrawal latency was detected by a motion detector. A maximal cutoff of 33 s was used to prevent tissue damage. Data are expressed as the mean withdrawal threshold  $\pm$  S.E.M.

### Tactile stimulation and FOS immunohistochemistry

On days 3, 5 and 10 after SNL, awake rats were gently restrained using a towel and received repetitive non-noxious tactile stimulation of the hindpaw ipsilateral to the SNL or sham surgery as previously described (Ma and Woolf, 1995). Two other groups of rats were studied: one group receiving tactile stimulation without any previous surgical manipulations and one group with ligation but with no tactile stimulation. The tactile stimulation consisted of gently rubbing the plantar surface of the hindpaw with the experimenter's thumb for a period of 2 s ("on") followed by an interval of 2 s ("off") for a total stimulation time of 10 min in accordance with previous procedures (Ma and Woolf, 1995; Catheline et al., 1999, 2001). After the stimulation the rats were returned to their home cages. All stimulation was done by an experimenter who was blind to the experimental group assignments. Two hours after tactile stimulation, animals were perfused transcardially with 50 ml of heparinized saline at 37 °C followed by 500 ml of cold (4 °C) 4% paraformaldehyde solution (pH 7.4). The spinal cords were removed carefully and postfixed in 4% paraformaldehyde solution at room temperature for 4 h, then were placed in 30% sucrose–phosphate buffer solution (PBS) overnight for cryoprotection. The L4–L6 segments of the spinal cord were embedded in OCT cryoprotectant compound (Allegiance Health Care Corp, Torrance, CA, USA) and stored at –20 °C until processed. Tissue blocks were cut in 30- $\mu$ m-thick coronal sections on a cryostat, and alternate sections were collected in PBS (pH 7.4). The free-floating sections were incubated in 5% normal goat serum (NGS) with Triton 100-X for 30 min at room temperature. The sections were incubated in anti-FOS antibody (1:10,000; Oncogene, San

Diego, CA, USA) diluted in 2% NGS/Triton overnight at room temperature. The following day, sections were rinsed in PBS and they were incubated in secondary antibody CY-3-conjugated goat anti-rabbit (1:500; Jackson Laboratories, West Grove, PA, USA) for 3 h at room temperature. Sections were rinsed in PBS, mounted on gelatin coated slides and coverslipped. As a negative staining control, sections were processed for immunohistochemistry with the primary antibody deleted. All sections from all of the groups were processed at the same time with the same solutions in wire bottom trays, assuring that all tissues received similar exposure to all reagents. Digital images were captured with a Nikon E800 fluorescence microscope equipped with standard filters for immunofluorescence. Images were acquired with a Hamamatsu C5810 color CCD camera and its proprietary Image Processor software (Hamamatsu Photonic Systems, Bridgewater, NJ, USA). All images were taken with the Hamamatsu camera set with the same gain, exposure and contrast settings. These settings were established manually until FOS immunoreactive profiles were clearly visible and remained unchanged throughout the course of the experiments. FOS-positive cells were manually counted from each image taken for laminae I–II (superficial dorsal horn) and laminae III–V (deep dorsal horn) of the spinal dorsal horn. Labeled nuclei were counted only when structures of the appropriate size and shape demonstrated clear increases in immunoreactivity when compared with the background level. All counting was performed by an individual blinded to the experimental treatments of the sections. The mean number of labeled cells per section was determined from 10 randomly selected sections through the L5 segment for each animal. Fluorescent images were converted to black and white images using the inversion function of the Adobe Photoshop software (version 6.0; Adobe Systems, San Jose, CA, USA) to illustrate FOS individual profiles more clearly.

### Statistical analysis

Significant differences within each experimental group for the behavioral tests over time were detected by one-factor ANOVA followed by the Fisher's least significant difference post hoc test. Two-factor ANOVA was used to detect significant differences in evoked FOS expression among treatment groups and across time. Significant differences among groups within each time period were detected by one-factor ANOVA followed by the Fisher's least significant difference post hoc test. Student's *t*-test was used for pair-wise comparisons. Significance was set at  $P < 0.05$ .

## RESULTS

### Microinjection sites

Histological verification at the end of the experiments demonstrated the injection sites and cannula tracks as shown

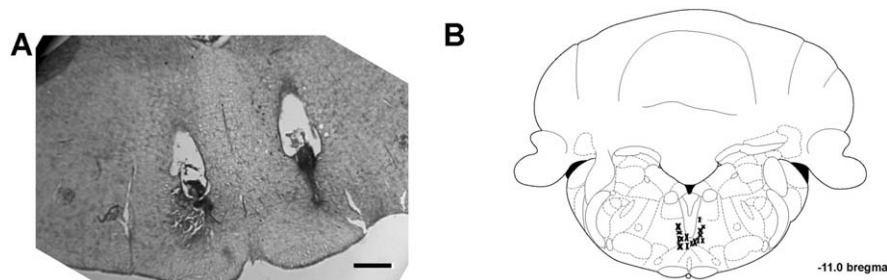
in Fig. 1. These microinjection sites have previously been shown to reduce the mRNA signal for the mu opioid receptor in the RVM and surrounding areas including the nucleus gigantocellularis (NGC) (Porreca et al., 2001).

### Dermorphin–saporin microinjection and behavioral hypersensitivity

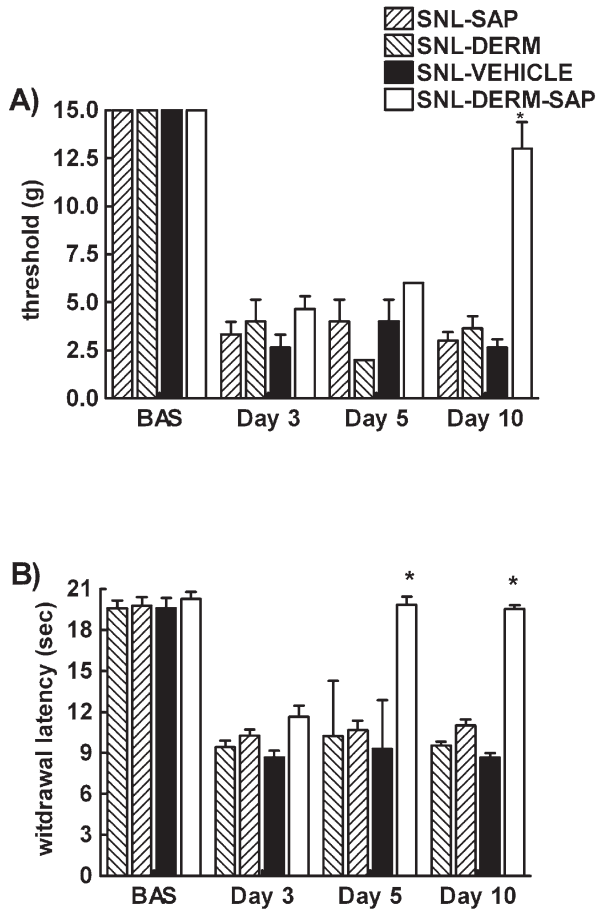
Rats ( $n=8$  per group) received a single bilateral microinjection of distilled water ( $0.5 \mu\text{l}$ ), dermorphin (3 pmol), saporin (3 pmol), or dermorphin–saporin conjugate (3 pmol) into the RVM. Our previous investigations revealed that this protocol of dermorphin–saporin treatment elicited a loss of RVM neurons expressing the  $\mu$ -opioid receptor at postinjection day 28 (Porreca et al., 2001; Burgess et al., 2002). Baseline sensory thresholds to von Frey filaments or to noxious thermal stimuli taken before ligation surgery did not differ significantly among groups of rats receiving RVM pretreatment 28 days earlier with distilled water, dermorphin, saporin or dermorphin–saporin (Fig. 2).

SNL rats had significantly reduced mechanical withdrawal thresholds (Fig. 2A) by 3 days post-SNL surgery, with withdrawal thresholds ranging from  $2.6 \pm 0.6$  to  $4.6 \pm 0.6$  g; these levels were significantly different from the baseline threshold of 15.0 g ( $P < 0.05$ ). Mechanical thresholds remained well below baseline thresholds 5 days post-surgery, in the range of  $4.0 \pm 1.1$  to  $6.0 \pm 0.1$  g. RVM microinjections of either saporin, dermorphin or vehicle did not alter the expected SNL-induced tactile hypersensitivity. Rats receiving microinjection of dermorphin–saporin into the RVM continued to demonstrate significant tactile hypersensitivity at the 5 day time point post-SNL; tactile thresholds were not significantly different from groups pretreated with RVM vehicle, saporin or dermorphin. The finding of continued tactile hypersensitivity in SNL animals pretreated with RVM dermorphin–saporin was expected based on previous observations demonstrating a differential time course of reversal of SNL-induced tactile and thermal hypersensitivity by RVM dermorphin–saporin (Burgess et al., 2002). At day 10 after SNL, animals injected with dermorphin–saporin had a mechanical threshold of  $13.0 \pm 1.4$  g, a value not different from pre-SNL values.

Animals receiving RVM microinjections of saporin, dermorphin, dermorphin–saporin or vehicle showed equivalent thermal responses following sham surgeries. SNL sur-



**Fig. 1.** Histological verification of microinjection sites into the RVM is shown by a representative photomicrograph of the cannula tracks in a Cresyl Violet-stained section (A). In (B), a diagram shows the sites of injections as reconstructed from histological sections. The diagram was adapted from Paxinos and Watson (1986). Scale bar=50  $\mu\text{m}$ .



**Fig. 2.** Tactile hypersensitivity was present on day 3 after SNL and persisted through day 5 (panel A). Baseline (BAS) paw withdrawal thresholds were measured 28 days after RVM microinjections but before SNL. Rats with microinjection of dermorphin-saporin (DERM-SAP) ( $n=8$ ) into the RVM showed reduced tactile hyperesthesia only at day 10 after SNL (\*  $P \leq 0.05$  vs. day 3 and day 5). Rats with microinjections in the RVM of SAP, DERM or vehicle ( $n=8$  per group) did not show reduced tactile hypersensitivity at any of the time points studied (panel A). Thermal hypersensitivity was demonstrated by reduced withdrawal latencies to radiant heat on day 3 after SNL surgery (panel B). Rats with microinjections of DERM-SAP into the RVM demonstrated reduced thermal hypersensitivity at days 5 and 10 after SNL (\*  $P < 0.05$  vs. day 5). Rats with microinjections of SAP, DERM and vehicle did not show reduced thermal hypersensitivity at any of the time points studied (panel B).

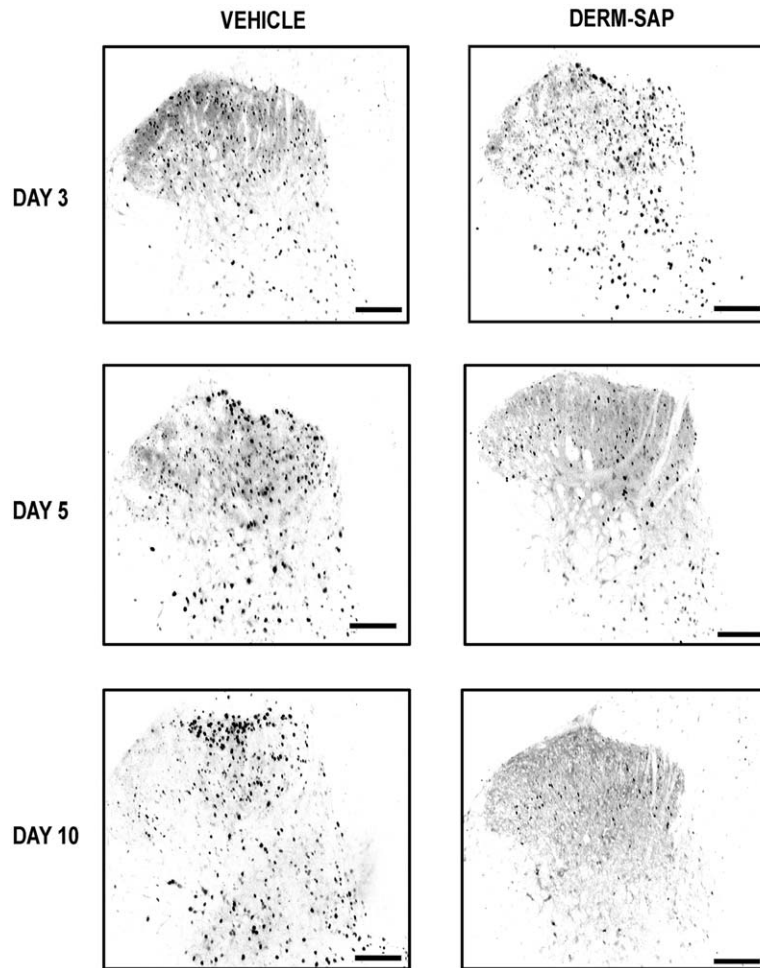
gery induced thermal hypersensitivity in all groups within 3 days of surgery (Fig. 2B). At this time-point, thermal withdrawal latencies were significantly reduced from a baseline range of  $19.5 \pm 0.7$  to  $20.3 \pm 0.5$  s before SNL or sham surgery to a range of  $8.6 \pm 0.4$  to  $11.6 \pm 0.8$  s after SNL surgery ( $P < 0.05$ ). Paw withdrawal latencies in SNL rats pretreated with RVM saporin, dermorphin or vehicle remained lower than baseline at post-surgery day 5 (range of  $9.3 \pm 3.5$  to  $10.2 \pm 4.0$  s), thresholds which were significantly different from sham-operated controls ( $P < 0.05$ ). Thermal thresholds of animals receiving microinjection of dermorphin-saporin into the RVM showed a full return to baseline values in withdrawal latency of  $19.8 \pm 0.5$  s by day 5, a value which did not differ significantly from pre-SNL

baseline. At day 10, the paw withdrawal latency for animals with microinjection of dermorphin-saporin was  $19.5 \pm 0.3$  s, also not different from pre-SNL baseline. The time-course of RVM dermorphin-saporin reversal of SNL-induced thermal hypersensitivity is consistent with our previous observations (Burgess et al., 2002).

#### Evoked FOS expression by tactile non-noxious stimulation

Separate groups of sham-operated and nerve-injured rats pretreated 28 days earlier with microinjections of vehicle ( $n=12$ , sham;  $n=12$  SNL), dermorphin ( $n=12$ ;  $n=12$  SNL), saporin ( $n=12$ , sham;  $n=12$  SNL) or the dermorphin-saporin conjugate ( $n=12$  sham;  $n=12$  SNL) into the RVM were tested 3, 5 and 10 days ( $n=4$  per group per time point) after surgery in order to evaluate touch-evoked FOS expression in the spinal cord (Fig. 3). Gentle stroking of the hindpaw of the sham-operated groups produced little FOS expression in the superficial lamina (I and II) and the deeper lamina (III and IV) of the dorsal horn. The level of FOS expression did not change over the 10 day testing period, and none of the pretreatments affected touch-evoked expression of FOS in the sham-operated groups. The mean values obtained from all the sham-operated groups over the entire time-course of the study ranged from a low of  $3.3 \pm 1.1$  FOS-labeled cells per section to a high of  $7.3 \pm 0.9$  FOS-labeled cells per section for the superficial lamina (Fig. 4). The comparable values for the deeper laminae ranged from  $9.5 \pm 0.7$  to  $13.8 \pm 0.9$  FOS-labeled cells per section (Fig. 4). Groups of rats which received SNL without tactile manipulation and rats receiving tactile stimulation without injury demonstrated negligible number of FOS expressing cells in the spinal cord dorsal horn; mean values for the ligated group with no stimulation and the stimulated group without stimulation were  $1.4 \pm 0.2$  and  $0.8 \pm 0.1$  FOS-labeled cells per section respectively (data not shown).

An increase in touch-evoked FOS-positive cells was seen in the SNL groups pretreated with RVM microinjections of vehicle, saporin and dermorphin at all time points. On post-SNL day 3, animals pretreated with RVM saporin showed  $17.7 \pm 1.8$  FOS positive cells vs.  $3.2 \pm 1.0$  in sham rats ( $P < 0.05$ ) in the ipsilateral superficial dorsal horn. Similarly, SNL animals pretreated with dermorphin or vehicle respectively showed  $18.0 \pm 0.9$  FOS positive cells vs.  $3.7 \pm 1.2$  in sham-operated rats ( $P < 0.05$ ) and  $15.5 \pm 1.6$  FOS positive cells vs.  $6.7 \pm 1.1$  in superficial ipsilateral dorsal horn ( $P < 0.05$ ). In deep dorsal horn, animals pretreated with RVM saporin showed  $49.5 \pm 1.1$  FOS positive cells vs.  $9.5 \pm 0.6$  in sham rats. Similarly, SNL rats pretreated with dermorphin and saporin showed  $45.7 \pm 1.4$  and  $55.7 \pm 1.2$  FOS positive cells respectively in deep dorsal horn. This was significantly different from the number of FOS positive cells in sham rats ( $8.2 \pm 0.8$  for dermorphin and  $10.5 \pm 0.6$  for saporin). Similar results were seen when FOS expression was evaluated at post-SNL days 5 and 10 in animals pretreated with RVM vehicle, dermorphin or saporin in the ipsilateral dorsal horn (Fig. 4). However, in the contralateral spinal cord dorsal horn, there were no

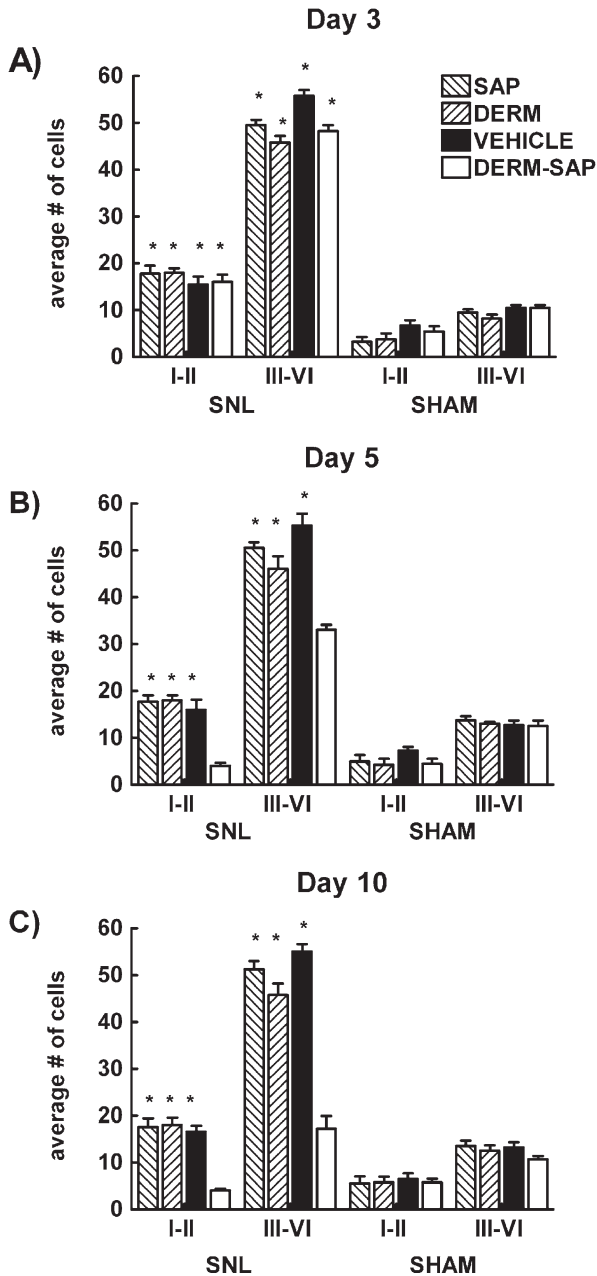


**Fig. 3.** Photomicrographs illustrating FOS-immunoreactive labeled profiles in coronal sections (30- $\mu$ -thick) of the lumbar cord at L5 are shown for animals with SNL and microinjection of either vehicle or dermorphin–saporin in the RVM. The images shown are black and white versions of the original fluorescent images obtained using Adobe Photoshop. The different time points after SNL are shown, illustrating the gradual reversal of SNL-induced enhanced touch-evoked expression of FOS. FOS expression did not change at any time point in animals receiving vehicle microinjection in the RVM. Scale bar=50  $\mu$ m.

significant changes in the expression of touch-evoked FOS over the time-course of the study. The mean values from the contralateral side over the entire time course ranged from a low of  $4.3 \pm 0.3$  to a high of  $7.3 \pm 0.9$  FOS-labeled cells per section in the superficial laminae. The comparable values for the deeper laminae ranged from a low of  $12.3 \pm 0.7$  to a high of  $15.3 \pm 1.3$  FOS-labeled cells per section (data not shown).

Animals receiving dermorphin–saporin microinjection into the RVM followed by SNL also showed a significant change in FOS expression after tactile stimulation when compared with sham-operated rats at the day 3 time point. In superficial dorsal horn,  $16.0 \pm 1.5$  FOS positive cells were detected in SNL vs.  $5.5 \pm 1.0$  in sham rats ( $P < 0.05$ ). In deep dorsal horn,  $48.2 \pm 1.2$  FOS positive cells were detected in SNL vs.  $10.5 \pm 0.6$  in sham rats ( $P < 0.05$ ). In contrast, at post-SNL days 5 and 10, only animals with previous microinjection of dermorphin–saporin demonstrated a time-dependent reduction in touch-evoked FOS expression. On post-SNL day 5, animals with RVM der-

morphin–saporin pretreatment showed  $4.0 \pm 0.7$  FOS positive cells in the ipsilateral superficial dorsal horn. This number was not significantly different from the number counted in sham-operated controls ( $4.5 \pm 1.0$  cells). This value was significantly less than the number of FOS-positive cells counted in SNL animals pretreated with saporin, dermorphin or vehicle. In the deep ipsilateral dorsal horn SNL animals pretreated with dermorphin–saporin showed  $33.0 \pm 1.0$  FOS positive cells which were significantly different from the sham group ( $12.5 \pm 1.1$  cells). This number was not significantly different from the other control groups (dermorphin, saporin and vehicle). On day 10, SNL animals with dermorphin–saporin injections had  $4.0 \pm 0.4$  cells in the ipsilateral superficial dorsal horn, a value not different from sham-operated controls ( $5.7 \pm 0.8$  cells,  $P < 0.05$ ), but significantly different from numbers of FOS-positive cells in animals pretreated with RVM saporin, dermorphin or vehicle. Similar results were obtained in the deep dorsal horn as SNL animals pretreated with dermorphin–saporin showed  $17.2 \pm 2.6$  FOS positive cells vs.  $10.7 \pm 0.6$



**Fig. 4.** The number of FOS-positive cells in the spinal cord dorsal horn at the L5 level counted in the side ipsilateral to the nerve injury is represented in a bar graph. Touch-evoked FOS expression was minimal in animals with sham-surgery at all time points. Animals with SNL presented a significant increase in numbers of FOS-positive cells after tactile stimulation (A) when compared with sham animals ( $* P < 0.05$ ). Microinjection of DERM-SAP into the RVM attenuated the increase of FOS-positive cells seen after tactile stimulation of the hindpaw ipsilateral to the side of injury on day 5 (B) when compared with sham animals ( $* P < 0.05$ ). The level of FOS-positive cells on day 10 in animals receiving microinjection of DERM-SAP was decreased from day 5 and it was no different to levels from sham animals ( $* P < 0.05$ ) (C). ( $n = 4$  per group per time point) (DERM, dermorphin; SAP, saporin).

cells in the sham group. There were significant differences between the number of FOS positive cells in the deep dorsal horn between the group pretreated with

dermorphin-saporin and the control groups (dermorphin, saporin and vehicle).

## DISCUSSION

In the present study, repetitive non-noxious tactile stimulation of the hindpaw produced an increase in the number of FOS-positive cells in the ipsilateral spinal dorsal horn of animals that had received SNL when compared with FOS expression in animals that received sham surgery. The increase in FOS-positive cells was seen in both the superficial and deep dorsal horn laminae and correlated in time with the expression of nerve injury-induced mechanical and thermal hypersensitivity, behaviors typical of the SNL model. These observations replicate previous reports of enhanced, touch-evoked FOS expression in the spinal dorsal horn following peripheral nerve injury (Catheline et al., 1999, 2001; Hao et al., 2003a,b) and collectively support previous suggestions that peripheral nerve injury elicits a state of "central sensitization" which may underlie, in part, exaggerated responses to afferent stimuli.

The immediate early gene c-fos is rapidly and transiently expressed in excited neurons in response to strong stimuli (Hunt et al., 1987; Willis and Coggeshall 1991). Numerous studies demonstrated that various noxious stimuli can induce c-fos resulting in expression of the FOS protein (Bullitt, 1990; Harris, 1998). In models of inflammation, tactile stimulation of the affected limb produces increased numbers of FOS-positive cells in the spinal cord dorsal horn relative to non-inflamed controls (Abbadie and Besson, 1993; Ma and Woolf, 1995; Wei et al., 1999a). Likewise, tactile stimulation also produces increased numbers of FOS-positive cells in the dorsal horn after SNL and after chronic constriction of the sciatic nerve (Catheline et al., 1999, 2001; Hao et al., 2003a,b). These observations suggest the presence of a state of central sensitization which may be directly responsible for the exaggerated neuronal response to some types of afferent stimuli. Consistent with this suggestion, blockade of spinal NMDA receptors, which are critical to central sensitization (Chapman et al., 1995), blocks both behavioral hypersensitivity as well as increased FOS expression in injury states.

Substantial evidence supports the concept that descending pain modulatory systems from the medulla play an important role in the expression of injury-induced behaviors. Numerous observations have shown that nociception-induced spinal FOS expression is attenuated by manipulations that are antinociceptive (Jones, 1992; Lee and Beitz, 1992; Pertovaara et al., 1993; Elliott et al., 1995; Honore et al., 1995). Spinal FOS expression elicited by noxious stimuli is reduced by activation of descending inhibition (Jones and Light, 1990; Gogas et al., 1991), or enhanced by spinal lesions which block tonic descending inhibition (Ren and Ruda, 1996; Wei et al., 1998). Selective lesions of supraspinal nuclei have shown differential effects on evoked FOS expression in inflammatory pain states. Lesions of the nucleus raphe magnus (NRM) with the serotonergic neurotoxin 5,7-dihydroxytryptamine or of the locus coeruleus/subcoeruleus (LC/SC) with the norad-

renergic neurotoxin DSP-4 resulted in increased FOS and thermal hyperalgesia in rats with inflammation induced by complete Freund's adjuvant (CFA) (Wei et al., 1999b). Conversely, lesions of the NGC with the soma-selective neurotoxin ibotenic acid resulted in a reduction in both FOS expression and thermal hyperalgesia in rats with CFA-induced inflammation (Wei et al., 1999b). It was interpreted that the NRM and LC/SC were sources of descending inhibitory pathways whereas the NGC was a source of descending facilitation of nociceptive inputs, and that enhanced or diminished perception of nociception may represent the interplay among these supraspinal loci (Wei et al., 1999a,b). It was concluded by these investigators that peripheral inflammation is associated with a facilitation of neuronal activity within the superficial laminae through descending pathways traveling in the DLF (Wei et al., 1999a). While the specific role of supraspinal nuclei in nerve injury-induced pain remains unknown, experimental nerve injury is completely abolished by lidocaine injection in the RVM suggesting a predominant influence of descending facilitation to maintain the state of central sensitization (Pertovaara et al., 1996; Kovelowski et al., 2000). In the present study, FOS expression was used as a measure of central sensitization after nerve injury. As noted above, FOS expression after peripheral injury can be modified by descending influences. The novel concept this study confers is that FOS expression can be correlated to the presence of mechanical and thermal hypersensitivity and therefore measuring FOS as a marker of central sensitization allows the analysis of the neuronal substrate of behavioral hypersensitivity and how descending modulation is able to facilitate neuronal activity at the spinal cord level.

Considerable evidence has accumulated to implicate the RVM as a critical, and perhaps convergent, node in the neuroanatomical pathways that enhance the sensitivity of the spinal dorsal horn to nociceptive inputs. The microinjection of lidocaine into the RVM abolished behavioral signs of enhanced pain after nerve injury or inflammation (Mansikka and Pertovaara, 1997; Pertovaara, 1998; Kovelowski et al., 2000). Moreover, lidocaine in the RVM abolished the increased sensitivity and firing rate, and the decreased stimulation threshold of dorsal horn WDR neurons sensitized by mustard oil-induced peripheral inflammation (Mansikka and Pertovaara, 1997; Pertovaara, 1998; Kovelowski et al., 2000). Likewise, hyperesthesias to tactile or thermal stimuli applied to animals with inflammation or peripheral nerve injury were abolished by chemical ablation of the RVM or physical disruption of its descending tracts in the DLF (Wei et al., 1998, 1999a,b; Ossipov et al., 2000).

The RVM neurons that are directly hyperpolarized by opioids, and therefore express the mu-opioid receptor, are hypothesized to be a primary source of descending pain facilitatory systems (Heinricher and McGaraughty, 1997; Heinricher et al., 2001, 2003; Heinricher and Neubert, 2004). Accordingly, the selective reduction in this population of RVM neurons by microinjection of dermorphin-saporin conjugate also abolished behavioral and neuro-

chemical endpoints indicative of injury-induced facilitation, including hyperesthesias to tactile and thermal stimuli, up-regulation of spinal dynorphin content and the enhanced capsaicin-evoked release of CGRP (Porreca et al., 2001; Burgess et al., 2002; Gardell et al., 2003). It should be noted that these effects of dermorphin-saporin occurred without affecting baseline sensory thresholds, suggesting that this population of cells may be activated as a consequence of injury or other stimuli (Mason, 2001). This evidence argues also against the possibility of indirect effects of microinjection of dermorphin-saporin into the RVM. Indeed, the behavioral endpoints of sensitization were not abolished during the initial 5 days after SNL, but were reversed over several days indicating the time-dependency of the descending facilitatory drive (Burgess et al., 2002; Gardell et al., 2003). Similar observations were made in rats with SNL and DLF lesions (Burgess et al., 2002; Gardell et al., 2003).

In the present study, repeated gentle touch to the hindpaw increased expression of FOS at 3, 5 and 10 days after induction of SNL in groups of animals pretreated with RVM vehicle, dermorphin, or saporin. In contrast, on days 5 and 10 after SNL, lesion of cells believed to play an important role in descending pain facilitation prevented the enhanced touch-evoked expression of FOS in the spinal dorsal horn of animals with peripheral nerve injury, suggesting a requirement of descending pain facilitation in order for nerve injury-induced central sensitization to be maintained. These findings are consistent with previous observations of SNL-induced behavioral hypersensitivity (Burgess et al., 2002). An interesting feature of those studies was the differential time course of reversal of SNL-induced tactile and thermal hypersensitivity by the dermorphin-saporin pretreatment, with the thermal hypersensitivity showing full reversal by day 5 post-SNL while the tactile hypersensitivity was maintained at post-SNL day 5 and reached full reversal by day 10. These findings were confirmed in the present studies where SNL animals pretreated with RVM dermorphin-saporin showed a full blockade of thermal, but not tactile, hypersensitivity on day 5 post-SNL, a time at which there was only a partial reduction in touch-evoked FOS expression. These findings support the possibility that the mechanisms underlying hypersensitivity to stimulation with von Frey filaments differ from those mediating thermal hypersensitivity. Differential effects of descending modulatory systems on specific sensory modalities have been previously noted (Burgess et al., 2002; Pertovaara and Wei, 2003). It is also interesting to note that there was only partial attenuation of FOS expression in deeper lamina on day 5, and this correlated with the tactile hypersensitivity still present at this time point. Tactile hypersensitivity has been correlated with FOS expression in these deep laminae (Ma and Woolf, 1995). On day 10 after SNL, both mechanical and thermal hypersensitivity were fully reversed and these results correlated with the full reversal of enhanced touch-evoked FOS expression. These results provide an anatomical correlation at the spinal cord level of the behavioral hypersensitivity seen after nerve injury.

Although a role of primary afferent discharge is well established as a mechanism driving neuropathic pain (Devor, 1991; Liu et al., 2001; Amir et al., 2002), the temporal relationship between sustained afferent inputs thought to underlie states of central sensitization and the manifestation of tactile and thermal hyperesthesias in response to nerve injury remains unresolved. Recent studies suggest that the correlation between nerve injury-induced ectopic discharge is strong at early, but not late, time points after injury (Sun et al., 2005). This possibility is consistent with our previous work which indicates that the behavioral features of nerve injury are maintained, but not initiated, by descending facilitatory influences from the RVM suggesting that peripheral afferent drive alone may not be sufficient to maintain injury-induced hypersensitivity. Additionally, some spinal neuroplastic changes associated with peripheral nerve injury, such as upregulation of dynorphin, also depend upon descending facilitatory influences from the RVM (Burgess et al., 2002; Gardell et al., 2003). In the present studies, the ablation of mu opioid receptor expressing cells in the RVM prevented the enhanced expression of FOS produced by repetitive gentle touch in animals with nerve injury, suggesting a requirement of descending facilitation from the RVM in maintaining a sensitized state of the spinal dorsal horn. The time-dependency of the effects of dermorphin–saporin also suggests that there is likely to be an ascending input that informs the RVM of the presence of nerve injury. The importance of ascending/descending spinal loops has been previously shown (Suzuki et al., 2002) and emphasizes the possible importance of NK-1 receptor expressing cells in the spinal dorsal horn (Suzuki et al., 2002; Vera-Portocarrero et al., submitted for publication). It is also possible that the dorsal column pathways may play an important role in this process as previously shown for behavioral features of neuropathic pain (Sun et al., 2001; Ossipov et al., 2002).

Other studies have demonstrated that afferent inputs are necessary for the expression of behaviors indicative of neuropathic pain even during the RVM-driven phase of sensitization. Lidocaine applied to the site of sacral nerve ligation 5 days after surgery reversibly abolished tactile and thermal hyperesthesias of the hindpaw but did not produce antinociception to noxious radiant heat and did not alter motor function (Malan et al., 2000). Transection of the sciatic nerve produced increased FOS expression in the dorsal horn of the spinal cord (Chi et al., 1993a,b). Conduction block of the sciatic nerve with bupivacaine administered immediately prior to or 2 days after transection decreased FOS expression in the superficial laminae and lamina V, but not in lamina III and IV (Chi et al., 1993a,b). In contrast, bupivacaine block administered 14 days after transection produced a marked reduction in FOS expression in all laminae of the spinal dorsal horn (Chi et al., 1993b). Central sensitization of spinal dorsal horn neurons determined by electrophysiologic methods in rats with CCI also demonstrated that local anesthetic block of the sciatic nerve also abolished sensitization (Sotgiu et al., 1996). Finally, dorsal rhizotomy or bupivacaine administration at L5 and L6 abolished behavioral signs of neu-

ropathic pain in rats with SNL 5 days after the injury (Yoon et al., 1996). The findings suggest that both descending pain facilitation and peripheral afferent input are essential to the maintenance of a state of central sensitization following peripheral nerve injuries. In the present studies, tactile and thermal hyperesthesias along with enhanced touch-evoked FOS expression occurred within 3 days of SNL in all groups. Consequently, the initial appearance of behavioral endpoints indicative of neuropathic pain along with enhanced touch-evoked FOS expression is likely due to mechanisms other than descending facilitation and is likely to be related primarily to enhanced afferent inputs which may drive the early phase of sensitization. As time-dependent neuroplastic adaptations occur, processes of descending facilitation may be engaged and then become a key mechanism which maintains spinal sensitization. Behavioral features of experimental neuropathic pain and FOS expression are not observed in those animals where descending facilitation has been abolished suggesting that whatever level of afferent input to the spinal cord remains at these later time points is not sufficient to maintain a sensitized state.

The results of the current investigation support the concept that behavioral signs of neuropathic pain are maintained by a state of spinal sensitization. Moreover, the study proposes the novel interpretation of spinal sensitization, as measured by increased FOS expression, following peripheral nerve injury is maintained by essential contributions of facilitatory spinopetal projections from the RVM and some level of afferent input. Additionally, our findings support the possibility of differences in mechanisms which underlie the expression of nerve injury-induced tactile and thermal hypersensitivity and point the integration of the latter response primarily at the spinal level. Taken together with previous findings which have demonstrated that nerve injury-induced upregulation of spinal dynorphin depends on descending pain facilitation systems, these data strongly suggest that the sustained neuroplastic adaptations in the spinal dorsal horn initiated by peripheral nerve injury are subsequently maintained by descending facilitation from RVM cells which express mu opioid receptors. Of particular importance may be the net influences of descending facilitation and plasticity in the brainstem with some sustained level of afferent input from injured nerve fibers (Han et al., 2000; Liu et al., 2000a,b; Sun et al., 2005) which may collectively be required to sustain central sensitization at later time points after peripheral nerve injury.

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