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Contribution of Afferent Pathways to Nerve-injury Induced Spontaneous Pain and Evoked Hypersensitivity

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Abstract

A predominant complaint in patients with neuropathic pain is spontaneous pain, often described as “burning”. Recent studies have demonstrated that negative reinforcement can be used to unmask spontaneous neuropathic pain allowing for mechanistic investigations. Here, ascending pathways that might contribute to evoked and spontaneous components of experimental neuropathic pain model were explored. Desensitization of TRPV1 positive fibers with systemic resiniferatoxin (RTX) abolished spinal nerve ligation (SNL) injury-induced thermal hypersensitivity and spontaneous pain, but had no effect on tactile hypersensitivity. Ablation of spinal NK-1 receptor expressing neurons blocked SNL-induced thermal and tactile hypersensitivity as well as spontaneous pain. Following nerve injury, upregulation of neuropeptide Y (NPY) is observed almost exclusively in large diameter fibers and inactivation of the brainstem target of these fibers in the n. gracilis prevents tactile, but not thermal, hypersensitivity. Blockade of NPY signaling within the n. gracilis failed to block SNL-induced spontaneous pain or thermal hyperalgesia while fully reversing tactile hypersensitivity. Moreover, microinjection of NPY into n. gracilis produced robust tactile hypersensitivity, but failed to induce conditioned place aversion. These data suggest that spontaneous neuropathic pain and thermal hyperalgesia are mediated by TRPV1 positive fibers and spinal NK-1 positive ascending projections. In contrast, the large diameter dorsal column projection can mediate nerve injury-induced tactile hypersensitivity, but does not contribute to spontaneous pain. As inhibition of tactile hypersensitivity can be achieved either by spinal manipulations or by inactivation of signaling within the n. gracilis, the enhanced paw withdrawal response evoked by tactile stimulation does not necessarily reflect “allodynia”.

Keywords

Ongoing pain; nerve injury; NPY; TRPV1; tactile allodynia; thermal hyperalgesia

Introduction

A common symptom of patients with neuropathic pain is spontaneous pain that is often described as burning [1; 2]. Some neuropathic pain patients also suffer from pain that is elicited by normally innocuous touch or cold, i.e., allodynia [12; 28]. Preclinical studies of

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experimental neuropathic pain have generally relied on enhanced withdrawal responses to normally innocuous tactile stimuli (i.e., von Frey filaments or brushing) or to noxious thermal stimuli. Following nerve injury, an enhanced response to a noxious thermal stimulus is a measure of hyperalgesia. However, whether an exaggerated response to a normally innocuous tactile stimulus is an indication of pain, i.e., “allodynia” has been questioned [13].

Systemic administration of resiniferatoxin (RTX), an ultra-potent TRPV1 agonist, to adult rats produces desensitization of TRPV1-positive fibers resulting in a long-lasting elimination of nerve-injury induced thermal hyperalgesia as well as thresholds to noxious heat; this treatment, however, does not affect nerve injury-induced tactile hypersensitivity [25]. Small unmyelinated fibers, including TRPV1-positive fibers synapse within the superficial dorsal horn to lamina I NK-1 positive cells and these have been demonstrated to be critical in expression of nerve-injury induced thermal and tactile hypersensitivity [21; 24; 32]. However, whether these mechanisms contribute to spontaneous pain is not known.

Nerve injury-induced tactile allodynia is thought to be mediated by large diameter A β fibers [40; 15; 6]. In addition to synapses in the spinal cord, however, these large diameter cells also project, via the dorsal column pathway, to brainstem nuclei including n. gracilis and n. cuneatus (for hindpaw and forepaw projections, respectively). Following spinal nerve ligation (SNL) injury, large diameter afferents selectively upregulate neuropeptide Y (NPY) [26]. Microinjection of NPY into the n. gracilis of uninjured rats elicits tactile, but not thermal hypersensitivity and inactivation of the n. gracilis following nerve injury with lidocaine or anti-NPY antiserum reverses tactile but not thermal hypersensitivity [31; 26; 42]. While the dorsal column pathway to n. gracilis contributes to enhanced withdrawal response following nerve injury, it is not known if this pathway contributes to neuropathic allodynia.

While “gain of function” responses to evoked stimuli are taken as the main translation feature of experimental neuropathic models [30], non-evoked, or spontaneous, pain is the most prominent feature of the neuropathic state [1]. Such pain has been difficult to measure preclinically [4; 30; 39; 18]. We recently demonstrated that nerve injury-induced spontaneous pain can be unmasked using the principle of negative reinforcement in models of partial nerve injury pain [18]. The present study explored the relative contribution of afferent and ascending sensory tracts to nerve-injury induced spontaneous pain and evoked hypersensitivity.

Materials and Methods

Male, Sprague Dawley rats (Harlan, Indianapolis, IN), 250–350 g at the time of testing, were maintained in a climate-controlled room on a 12 hr light/dark cycle (lights on at 7:00 A.M.) with food and water available *ad libitum*. All testing was 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 and received approval from the Institutional Animal Care and Use Committee of the University of Arizona.

RVM and n. Gracilis cannulation

Animals were anesthetized with injection of ketamine–xylazine (100 mg/kg, i.p.) and placed in a stereotaxic apparatus. Bilateral cannulation of the RVM was performed as previously described [3]. Two 26 gauge guide cannulas separated by 1.2 mm (Plastics One, Roanoke, VA) were directed toward the lateral portions of the RVM (anteroposterior, -11.0 mm from bregma; lateral, ± 0.6 mm; dorsoventral, -7.5 mm from the dura mater [27]). Cannulation of then. gracilis was performed as previously described [26]. A 26-gauge guide cannula

(Plastics One, Roanoke, VA) was directed towards the ipsilateral n. gracilis (anteroposterior, -5.5 mm from interaural line, mediolateral 0.5 mm from midline, and 2 mm above the interaural line [27]). Both RVM and n. gracilis guide cannulas were cemented in place and secured to the skull by small stainless steel machine screws. Rats then received gentamicin and were allowed to recover 7 days before any behavioral testing and SNL or sham surgeries. Shorter recovery periods were avoided to prevent possible behavioral impairment in place conditioning assays.

Sar⁹Met(O₂)¹¹ Substance P-Saporin (SSP-saporin) administration

Rats underwent surgery for implantation of an intrathecal catheter under halothane anesthesia (polyethylene-10 tubing; 7.5 cm) as described previously [41] for drug administration at the level of the lumbar cord. Rats were anesthetized with ketamine: xylazine (Sigma, 80mg/kg:20mg/kg) and the atlanto-occipital membrane was exposed and punctured. A section of PE-10 tubing 8 cm in length was passed caudally from the cisterna magna to the lumbar enlargement. Intrathecal injections of blank-SAP (Advanced Targeting Systems, 0.1 μ M, 16.5 pg/5 μ l) or the substituted SP analog, Sar⁹Met(O₂)¹¹ Substance P-Saporin (SSP-SAP, Advanced Targeting Systems, 0.1 μ M, 16.5pg/5 μ l) were made in a volume of 5 μ l followed by a 9 μ l flush of saline. This dose is 10-fold lower than previously published data with unsubstituted SP-SAP [21], as Sar⁹Met(O₂)¹¹ Substance P-Saporin shows approximately 10-fold greater potency at the NK-1 receptor in addition to resistance to peptidase digestion [23]. Progress of the injection was monitored by the movement of an air bubble placed between drug solution and saline flush. Immediately following injection, catheters were slowly removed from the spinal cord and the wound closed. All rats received gentamicin following surgery. Any animals displaying motor impairment or paralysis during recovery (<10% total rats) were immediately euthanized. Animals were kept in home cages for 28 days prior to further testing to allow for elimination of NK-1 receptor expressing cells in the spinal cord as described previously [38]. Animals were routinely checked across the 28 days to monitor health.

Spinal Nerve Ligation

One week recovery after RVM or n. gracilis surgeries, baseline sensory thresholds were measured for evoked pain behaviors. The surgical procedure for L5/L6 spinal nerve ligation was performed according to that described previously [17]. Sham-operated control rats were prepared in an identical manner except that the L5/L6 spinal nerves were not ligated. The behavior of the rats was monitored carefully for any visual indication of motor disorders or change in weight or general health.

Behavioral testing

Tactile and thermal hypersensitivity and place preference assays were determined 14 days following SNL or sham surgery by experimenters blinded to the treatment groups of the rats.

Tactile hypersensitivity

Tactile hypersensitivity was determined 14 days following SNL or sham surgery by measuring withdrawal threshold of the ipsilateral hindpaw in response to probing with a series of eight calibrated von Frey filaments by sequentially increasing and decreasing the stimulus strength ("up and down" method), analyzed using a Dixon nonparametric test [7; 5].

Thermal hypersensitivity

Thermal hypersensitivity was determined 14 days following SNL or sham surgery by measuring withdrawal latency of the ipsilateral hindpaw in response to infrared radiant heat.

A motion detector halted both lamp and timer when the paw was withdrawn. Baseline latencies were established at 17-25 sec to allow a sufficient window for the detection of possible hyperalgesia. A maximal cutoff of 33 sec was used to prevent tissue damage.

Conditioned place pairing

The single trial conditioned place preference protocol was performed as previously described [18], with conditioning day 10 days following SNL or sham surgeries. Starting 7 days post-SNL/sham surgery, all rats underwent a 3 day pre-conditioning period with behavior recorded on day 3 to verify no pre-conditioning chamber preference. All animals are exposed to the environment with full access to all chambers across 30 min each day. On day 3, behavior was recorded for 15 min and analyzed to verify absence of preconditioning chamber preference. The following day (day 4), rats received the appropriate control (i.e. vehicle) and immediately placed in the appropriate conditioning chamber for 30 min. Four (4) hr later, rats received the appropriate drug treatment and immediately placed in the opposite conditioning chamber for 30 min. Chamber pairings were counterbalanced. On test day 5, 20 hours following the afternoon pairing, rats were placed in the CPP box with access to all chambers and their behavior recorded for 15 min for analysis for chamber preference. Increased post-conditioning time spent in the drug paired chamber, as compared to preconditioning time, indicates conditioned place preference. Decreased post-conditioning time spent in the drug paired chamber, as compared to pre-conditioning time, indicates conditioned place aversion. No change between time spent in the drug paired chamber, as compared to pre-conditioning time, indicates no conditioned place preference or aversion.

Resiniferatoxin injection

Resiniferatoxin (RTX, Tocris Bioscience), an ultrapotent TRPV1 receptor agonist, was administered systemically (0.1 mg/kg, i.p.) in a dose previously demonstrated to abrogate thermal responses across a period of 40 days, the longest time-point tested [25]. RTX was dissolved in a 95% ethanol mixture which was used as the vehicle control.

RVM and N. gracilis microinjection

Saline or lidocaine (4% w/v) were administered by slowly expelling 0.5 μ l through a 33 gauge injection cannula inserted through the guide cannula and protruding an additional 1mm into fresh brain tissue to prevent backflow of drug into the guide cannula. Drug administration into the n. gracilis was performed by slowly expelling 0.5 μ l volume of the anti-NPY antiserum, NPY (1 nmol, 2.66 μ g/0.5 μ l) or appropriate vehicle through a 33 gauge injection cannula inserted an additional 2 mm into fresh brain tissue to prevent backflow of drug into the guide cannula. Anti-NPY antiserum (Peninsula Laboratories, Inc., San Carlos, CA) was dissolved in 50 μ l distilled water. Pre-adsorbed serum was prepared from the anti-NPY antiserum as a control. The preadsorbed serum was prepared by incubating 0.2 ml of the antiserum (40 mg of protein/ml) with 0.8 ml of agarose-bound protein A (Vector Laboratories, Burlingame, CA) for 24 hr at 4°C. The suspension was pelleted by low-speed centrifugation (5000 $\times$ g for 5 min). The supernatant, which is devoid of anti-NPY IgG, was used to define the IgG-independent effects of the antiserum administration. Doses of the anti-NPY antiserum and NPY were based on previously published data [26].

Tissue verification

After the termination of all the behavioral testing, 0.5 μ l Indian ink was injected into the bilateral RVM or the n. gracilis. Rats were perfused transcardially with 0.9% 100-150 ml saline, followed by 10% formalin solution. Subsequent to perfusion, the brains were rapidly removed and placed into the same, fresh fixative to be fixed for additional 4 h at 4°C.

Subsequently, the brains were soaked in 25% sucrose solution overnight at 4°C and then cut serially into 30 µm thick coronal sections on a freezing microtome (Microm, HM 525; International GmbH, Germany). Sections were stained with cresyl violet to verify the RVM injection. Data from animals with incorrectly placed cannula were not included within the data analysis.

Immunohistochemistry

Rats were deeply anesthetized with a mixture of ketamine/xylazine and perfused transcardially with 100 ml saline, followed by 500 ml cold 4% paraformaldehyde. After perfusion the spinal cords were removed, post-fixed overnight in 4% paraformaldehyde, and cryoprotected with 25% sucrose in phosphate buffered saline (PBS) overnight at 4°C. Coronal frozen sections (20 µm) were cut from the lumbar enlargement of the spinal cord. Spinal cord sections were immunolabeled with a rabbit antiserum against the NK1 receptor (1:4000, Millipore). The sections were washed three times for 10 min each in PBS and then preincubated with PBS containing 5% normal goat serum (NGS) and 0.3% Triton X-100 for 30 min at room temperature. The sections were then incubated overnight in the primary antiserum diluted in 2% NGS. The following day the sections were washed three times in PBS for 10 min each, followed by incubation with secondary antibody (FITC-conjugated goat anti-rabbit IgG; 1:2000; Invitrogen) for 2 h. The sections were rinsed and mounted in Vectashield. Fluorescent digital images were captured using an Olympus BX51 microscope using a Hamamatsu CCD digital camera. The person taking the digital pictures was blinded to the experimental conditions.

Statistical Analysis

For analysis of evoked pain behaviors, significant changes from pre-surgery baseline control values were detected by ANOVA, followed by Bonferroni post-test. These evaluations were all performed using GraphPad Prism 5.03 (GraphPad Software, San Diego, CA, www.graphpad.com). For CPP experiments, data was analyzed before conditioning (baseline) and after conditioning using two-factor ANOVA (chambers vs. treatment) followed by Bonferroni test of post-conditioning compared to pre-conditioning time spent in the drug paired chamber to determine conditioned place preference (increase in post-conditioning time vs pre-conditioning time) or conditioned place aversion (decrease in post-conditioning time vs. pre-conditioning time). If significant conditioned place preference or conditioned place aversion was determined, group differences were analyzed using difference from baseline scores were calculated for each rat using the formula: test time in chamber - preconditioning time spent in chamber. Difference scores from baseline for the drug paired chamber between SNL and sham operated rats were analyzed using paired *t*-tests. For all analyses, significance was set at $P < 0.05$.

Results

Resiniferatoxin (RTX) desensitization of TRPV1 positive afferent fibers blocks SNL-induced spontaneous pain

Systemic administration of RTX has been demonstrated to produce long-term desensitization of TRPV1 positive fibers [34]. Rats showed thermal hyperalgesia (Fig 1a) and tactile hypersensitivity (Fig 1b) 5 days following SNL surgery. Consistent with our previous study [25], systemic administration of RTX (0.1 mg/kg, i.p.) 3 days prior to testing evoked behaviors eliminated responses to noxious thermal stimulation in sham as well as SNL treated rats (Fig 1a; *** $p < 0.001$ vs sham vehicle). Moreover, systemic RTX administration at the same dose failed to alter SNL-induced tactile hypersensitivity (Fig 1b; *** $p < 0.001$ vs sham vehicle).

Systemic RTX administration (0.1 mg/kg, i.p.) 5 days following SNL and 3 days prior to habituation blocked RVM lidocaine-induced place preference in SNL treated rats. Pre-conditioning times spent in the saline or lidocaine paired chambers were equivalent across all treatment groups ($p>0.05$). As no group differences were observed, data were pooled across groups for graphical representation (Fig 1c). SNL rats that received vehicle injection showed place preference to the chamber paired with RVM lidocaine (Fig 1c, $**p<0.01$ vs pre-conditioning) consistent with previous observations [18]. In contrast, SNL rats that were treated with systemic RTX showed equivalent time spent in the lidocaine- and saline-paired chambers (Fig 1c). Difference from baseline scores confirmed that only the SNL rats treated with vehicle showed increased time spent in the RVM lidocaine paired chamber, and that RTX treatment blocked preference for the RVM lidocaine paired chamber (Fig 1d, $***p<0.001$ vs sham vehicle).

SSP-SAP decreases the number of NK-1 receptor expressing cells in the spinal cord

SSP-SAP (1 nM, 1.65 pg/0.5 μ l) resulted in an almost total loss of NK1 receptor staining in the spinal dorsal horn 28-30 days after intrathecal administration (Fig. 2a). Spinal administration of equivalent concentration of blank-SAP (1 nM, 1.65 pg/0.5 μ l) did not alter NK1 receptor expression (Fig. 2a), indicating ablation of the NK-1 receptor expressing cells was specific to the saporin-conjugated selective NK-1 receptor agonist as previously demonstrated [23].

SSP-SAP blocks SNL-induced thermal hyperalgesia and tactile hypersensitivity

The paw withdrawal thresholds to probing with von Frey filaments and the withdrawal latencies from noxious radiant heat were determined 7 days following SNL or sham surgeries in animals treated 35 days previously with blank-SAP or SSP-SAP. Consistent with previous studies, pre-surgery thermal and tactile thresholds did not differ irrespective of treatment group ($p>0.05$, data not shown) [21]. Post-surgery sensory thresholds of sham rats did not differ from pre-surgical baselines irrespective of treatment group ($p>0.05$). In SNL rats treated with blank-SAP, robust thermal hyperalgesia and tactile hypersensitivity was observed (Fig 2b, c, d $***$ indicates $p<0.001$ vs pre-SNL). In contrast, thermal and tactile hypersensitivity failed to develop in SNL rats that had received spinal SSP-SAP treatment (Fig. 2b, c, $p>0.05$, vs pre-SNL), indicating that ablation of spinal NK-1 receptor expressing neurons blocked SNL-induced thermal hyperalgesia and tactile hypersensitivity as previously reported [24]. No differences in responses to thermal or tactile stimulation were observed in sham-operated animals irrespective of treatment group.

SSP-SAP blocks spontaneous pain in the SNL rats

To determine whether ablation of NK-1 receptor expressing neurons within the spinal cord also blocks SNL-induced spontaneous pain, rats previously treated with blank-SAP (control) or SSP-SAP underwent single trial conditioning with microinjection of lidocaine into the RVM, previously demonstrated to induce CPP in SNL rats [18]. Pre-conditioning times spent in the saline or lidocaine paired chambers were equivalent across all treatment groups ($p>0.05$). As no group differences were observed, data were pooled across groups for graphical representation (Fig 3a). Rats with SNL that had received intrathecal injection of blank-SAP showed a significant preference for RVM lidocaine paired chamber (Fig 3a, $*$ indicates $p<0.05$ vs pre-lidocaine). In contrast, SNL treated rats with SSP-SAP showed no preference for the RVM lidocaine paired chamber. Sham SNL rats had equivalent post conditioning times spent in the saline and lidocaine paired chambers irrespective of whether they received blank-SAP or SSP-SAP. Difference from baseline scores verified that SSP-SAP blocked RVM lidocaine induced CPP in the SNL rats, as only SNL rats with blank-SAP demonstrated increased time spent in the lidocaine paired chamber (Fig 3b, $*$ indicates $p<0.05$ vs SSP-SAP+SNL group).

NPY into the n. gracilis induces tactile hypersensitivity, but not CPA in uninjured rats

Consistent with previous reports [26], microinjection of NPY (2.66 $\mu\text{g}/0.5 \mu\text{l}$) into the n. gracilis induced robust tactile hypersensitivity that peaked within 20 min of administration and dissipated within 40 min post-administration (Fig 4a, *** $p < 0.001$, ** $p < 0.01$ vs. pre-drug BL). Administration of this same dose of NPY into n. gracilis, however, failed to induce conditioned place aversion (Fig 4b). These data indicate that administration of NPY into the n. gracilis of uninjured rats enhances paw withdrawal responses to tactile stimulation but is not aversive.

Anti-NPY into the n. gracilis reverses tactile hypersensitivity, but not spontaneous pain

Consistent with previous reports [26], microinjection of anti-NPY antiserum (20 $\mu\text{g}/0.5 \mu\text{l}$) reversed SNL-induced tactile hypersensitivity within 10 min lasting through 60 min post-injection and dissipating within 90 min post-injection (Fig 5a, * $p < 0.05$ vs post-surgery). Micro-injection of the same dose of anti-NPY antiserum failed to induce CPP in nerve injured rats. Pre-conditioning times spent in the saline or anti-NPY antiserum paired chambers were equivalent (Fig 5b, $p > 0.05$). Following conditioning, both sham and SNL treated rats spent equivalent time in the saline- and anti-NPY paired chambers, indicating no conditioned place preference to the anti-NPY paired chamber (Fig 5b, $p > 0.05$).

Discussion

The present study investigated the possible contribution of specific classes of afferent fibers and ascending pathways in spontaneous and evoked components of pain following experimental nerve-injury. Our findings demonstrate that (a) TRPV1 positive afferent fibers are critical for SNL-induced spontaneous pain and thermal hyperalgesia but may not be essential for hypersensitivity to a tactile stimulus; (b) spinal NK-1 positive projections mediate nerve injury-induced spontaneous pain as well as thermal hyperalgesia and tactile hypersensitivity; and (c) NPY-positive signaling within the n. gracilis mediates SNL-induced tactile hypersensitivity, but not spontaneous pain.

Consistent with previous studies, desensitization of TRPV1 positive fibers following systemic administration of resiniferatoxin eliminated the behavioral response to noxious thermal stimulation in both sham- and SNL-rats but failed to alter responses to tactile stimulation [25]. Here, functional blockade of TRPV1 positive fibers also blocked nerve-injury induced spontaneous pain. The possibility that spontaneous pain and touch-evoked allodynia are mediated by different fiber classes is supported clinically. Koltzenburg and colleagues found that compression of the radial nerve resulted in progressive block of large myelinated ($A\beta$) followed by thin myelinated ($A\delta$) fibers and resulted in an inhibition of brush-evoked pain without eliminating ongoing pain in patients with chronic neuralgia [20]. Notably, the brush-evoked pain was eliminated prior to loss of cold sensation indicating transmission by $A\beta$ fibers, which normally encode non-painful tactile stimuli. Additionally, pressure cuff blocks have demonstrated that abolishing $A\beta$ fiber function blocked allodynia while maintaining thermal sensation [12].

Human psychophysical studies indicate that activation of touch fibers produces sensations of pain when TRPV1 positive C-fibers are driven by capsaicin [37]. Additionally, touch can elicit pain in the setting of nerve injury [12; 20]. Patients with post-herpetic neuralgia (PHN) have been classified into subgroups characterized by those with prominent allodynia as well as others with impaired responses to touch and thermal stimuli and spontaneous pain [11]. In patients that have profound loss of thermosensory and nociceptor function, intradermal lidocaine failed to alleviate pain, leading to the proposal that pain in these patients is mediated by increased spontaneous activity in deafferented central neurons and/or

reorganization of central connections [11]. However, in patients with preserved sensation, application of intradermal local anesthetic produced pain relief [9, 27], leading to the proposal that this group of patients have neuropathic pain driven by “irritable nociceptors” [11]. Other studies show that selective stimulation of TRPV1 positive nociceptive fibers by application of capsaicin to an area of post-herpetic neuralgia (PHN) pain increased pain and allodynia [28]. Similarly, local anesthetic block of painful foci in patients with reflex sympathetic dystrophy (RSD) abolished mechano-allodynia, cold allodynia, and spontaneous pain while tactile perception was preserved [12]. Our studies are consistent with these observations in humans in suggesting that TRPV1 positive fibers likely mediate spontaneous neuropathic pain, often described clinically as “burning” pain as well as thermal responses. In neuropathic pain questionnaires, one of the strongest predictors of nerve injury pain is the description of pain as “burning” [2], an observation that implicates C-nociceptors in spite of the fact that not all patients experience thermal hyperalgesia [20]. It should be noted that the “burning” aspect of pain may reflect either dysfunction of injured nerves as would occur in a neuropathic state or physiological function of nerves in an inflammatory setting [2; 16; 10]. Preclinical studies have suggested that tactile allodynia may be mediated by A β fiber inputs to a sensitized spinal cord [6]. Our studies show that tactile hypersensitivity is present in SNL-rats even after functional desensitization of TRPV1-positive fibers, a finding that argues against interpretation of this behavioral response as “allodynia”.

Nociceptive fibers, such as TRPV1 positive fibers, have been demonstrated to form synaptic connections with NK-1 positive cells in spinal cord lamina I [36]. In the present studies, ablation of NK-1 receptor expressing cells was shown to eliminate all components of nerve-injury induced pain including thermal and tactile hypersensitivity as well as spontaneous pain. The NK-1 receptor has been suggested to be expressed on approximately 80% of the projection neurons in lamina I of the spinal cord that play a crucial role in the transmission of ascending nociceptive information to regions including the parabrachial area and thalamus [22; 35; 36]. Additionally, nerve injury has been suggested to engage spinal NK-1 receptor expressing cells as the ascending limb of a pronociceptive spinal-bulbo-spinal loop proposed to maintain spinal sensitization [33]. This pathway is also believed to drive descending pain facilitatory pathways from the RVM that are critical to the expression of nerve injury induced tactile and thermal hypersensitivity as well as spontaneous pain [29; 3; 38; 18]. Previous reports have shown that ablation of spinal NK-1 receptor expressing cells reliably block nerve-injury induced tactile and thermal responses [21; 24; 14] as well as SNL-induced increase in evoked firing of spinal neurons [32]. Importantly, ablation of spinal NK-1 expressing cells blocked SNL-induced increase in spontaneous activity of spinal dorsal horn neurons, suggesting a role for these neurons in SNL-induced spontaneous pain [32]; our present findings agree with this conclusion. Thus, converging evidence supports the importance of NK-1 receptor expressing cells in neuropathic spontaneous pain as well as in allodynia and thermal hyperalgesia.

Large diameter fibers encode touch and vibration through the ascending dorsal column pathway. Multiple studies have demonstrated that interference with this pathway blocks nerve-injury induced tactile, but not thermal, hypersensitivity. Spinal hemisection or dorsal column lesion blocked tactile hypersensitivity when performed ipsilateral, but not contralateral to the nerve injury [31]. Moreover, inactivation of the n. gracilis, the projection site of low threshold myelinated fibers from the hindpaw, blocks nerve-injury induced tactile hypersensitivity, but not thermal hyperalgesia [31; 26]. NPY is selectively upregulated in large diameter fibers and in the n. gracilis after nerve injury [26]. Consistent with our previous studies, blockade of NPY signaling in n. gracilis reversed nerve injury-induced tactile hypersensitivity. However, anti-NPY antiserum in n. gracilis at a dose that effectively blocked tactile hypersensitivity failed to produce place preference in nerve injured rats

suggesting that this pathway does not contribute to spontaneous pain. Additionally, microinjection of NPY into n. gracilis at a dose that produces robust tactile, but not thermal hypersensitivity, failed to produce conditioned place aversion. It should be noted that neuropathic pain patients often report unfamiliar sensations that are described as dysesthesias but that are not identified as “pain” [9]. Additionally, such patients have reported “unpleasant” feelings in response to tactile stimulation, but notably the sensation is not referred to as pain [19; 20; 9]. Thus, while enhancement of tactile responses through this pathway can be elicited pharmacologically with microinjection of NPY, this manipulation does not produce a state that is sufficiently aversive to be detected in this assay, as would be expected in pain. Nevertheless, the stimulation of signaling at this site might be hypothesized to contribute to “unpleasant” sensations as reported in patients.

As noted above, however, interference with spinal NK-1 positive pathways, as well as many other spinal manipulations (e.g., spinal administration of clonidine or ω -conotoxin [18]) blocks nerve injury-induced enhanced behavioral responses to tactile stimulation. Clinical studies have shown that spinal administration of adenosine can block allodynia without affecting ongoing pain [8]. Thus, enhanced behaviors to tactile stimulation following nerve injury can be modulated either through a spinal pathway or by interfering with the dorsal column (i.e., touch) pathway. These observations create confusion as to whether enhanced behavioral sensitivity to tactile stimulation can be interpreted as “allodynia”. Hogan and colleagues propose that stimulation by von Frey filaments produces sensations in the form of itch or tickle, which have been demonstrated to be highly motivating without being painful [13]. These conclusions are consistent with our findings that interference with, or stimulating, signaling within n. gracilis does not elicit preference in nerve injured animals or promote aversion in uninjured animals. Collectively, these observations suggest that the enhanced evoked hypersensitivity to touch stimuli is a complex behavior, possibly reflecting both aversive and non-aversive components that are associated with the neuropathic state.

While our data and clinical observations support the role of TRPV1 positive fibers in neuropathic pain, it is not clear if additional contributions may arise from TRPV1 negative fibers or if there is an additional contribution of mechanosensation to allodynia from “desensitized” TRPV1 positive fibers. The present study highlights the uncertainty of nerve-injury induced tactile hypersensitivity as a measure of clinical painful allodynia. Whereas spinal manipulations that reverse SNL-induced tactile hypersensitivity are likely to reflect painful allodynia, it is impossible to discern whether changes in threshold responses to tactile stimuli resulting from systemic treatments reflects reduction in painful allodynia or non-painful sensations. These studies also highlight the need for increased understanding of mechanisms by which spinal NK-1 positive cells drive pain. Increased understanding of such mechanisms may offer translational opportunities for the discovery of novel therapeutic strategies for neuropathic pain.

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Figure 1.

RTX eliminated SNL-induced thermal hyperalgesia and spontaneous pain, but not tactile hypersensitivity. A) Rats showed significant reduction in paw-flick latencies 7 days following SNL surgery (post-surgery). Systemic administration of RTX (0.1 mg/kg, i.p.) produced robust analgesia, increasing paw-flick latencies of both sham and SNL treated rats to near cut-off (32-s) values (post-RTX). *indicates, $p < 0.05$ vs pre-surgery, $n = 8$. B) Rats showed significant reduction in paw withdrawal thresholds to probing with von Frey filaments 7 days following SNL surgery (post-surgery). Systemic administration of RTX failed to alter SNL-induced tactile hypersensitivity (post-RTX). *indicates $p < 0.05$ vs. pre-surgery, $n = 8$. C) All rats showed equivalent pre-conditioning time spent in the conditioning chambers. As no differences were observed between treatment groups, pre-conditioning values were pooled for graphical representation. SNL rats treated with vehicle (SNL-Vehicle) showed increased time spent in the RVM lidocaine paired chamber following conditioning. Treatment with RTX prior to the CPP protocol blocked the lidocaine induced CPP in SNL treated rats. *indicates $p < 0.05$ vs. pre-conditioning, $n = 5-7$. D) Difference from baseline scores confirm that only SNL rats treated with vehicle demonstrated CPP to the RVM lidocaine paired chamber, and that RTX treatment 3 days prior to the start of CPP blocked the RVM lidocaine induced CPP. *indicates $p < 0.05$ vs. vehicle-sham treated rats, $n = 5-7$.

Figure 2. Spinal SSP-SAP ablation of NK-1 receptor expressing cells blocks SNL-induced evoked pain. A) Representative image showing immunofluorescent staining of NK-1 receptors within the spinal dorsal horn 40 days after spinal administration of Sar-SP saporin (SSP-SAP) or control injection (Blank-SAP). SSP-SAP treated rats showed greatly diminished NK-1 immunopositive staining. Animals that received the control injection (Blank-SAP) show clear labeling of the NK-1 receptor. B) SNL induced thermal hyperalgesia within 7 days in rats that received the control saporin injection (Blank-SAP) spinal Blank SAP 28 days prior to the SNL surgery. In contrast, SNL rats treated with SSP-SAP 28 days prior to SNL surgery failed to develop thermal hypersensitivity. SSP-SAP failed to alter paw withdrawal latencies of sham rats. C) SNL induced tactile hypersensitivity within 7 days in rats that received spinal Blank SAP 28 days prior to the SNL surgery. In contrast, SNL rats treated with SSP-SAP 28 days prior to SNL surgery failed to develop tactile hypersensitivity. All graphs are mean $\pm$ SEM, *** indicates $p < 0.001$ vs. pre-surgery values, $n = 12-18$.

Figure 3.

Spinal SSP-SAP ablation of NK-1 receptor expressing cells blocks SNL-induced spontaneous pain. A) Pre-conditioning time spent in the conditioning chambers did not differ across treatment groups, therefore data were pooled for graphical representation. RVM lidocaine did not produced CPP in sham operated rats irrespective of treatment group. SNL rats that received spinal control injection (Blank-SAP) 28 days prior to SNL surgery showed increased time spent in the lidocaine paired chamber, * indicates $p < 0.05$ compared to pre-conditioning values. SNL-rats that received spinal SSP-SAP injection 28 days prior to SNL surgery failed to show CPP to the lidocaine paired chamber. B) Difference scores confirm that only SNL rats that received spinal injection of the control (Blank-SAP) showed CPP to the lidocaine paired chamber, * indicates $p < 0.05$ vs. Sham-Blank control rats. All graphs are mean $\pm$ SEM, $n = 6-8$.

Figure 4.

NPY administration into the n. gracilis induced tactile hypersensitivity, but did not induce conditioned place aversion in naïve rats. A) Administration of NPY (2.66 µg/0.5 µl) into the ipsilateral n. gracilis induced robust tactile hypersensitivity within 20 min of administration that resolved by 40 min post-administration. Administration of saline failed to alter paw withdrawal thresholds. *indicates $p < 0.05$ vs. BL, $n = 9$. B) Administration of the same dose of NPY into the n. gracilis failed to alter time spent in conditioning chambers.

Figure 5.

Anti-NPY administration into the n. gracilis reversed SNL-induced tactile hypersensitivity, but not spontaneous pain. A) Administration of anti-NPY (20 μ g/0.5 μ l) into the ipsilateral n. gracilis fully reversed SNL-induced tactile hypersensitivity within 20 min, with thresholds returning to pre-anti-NPY values within 40 min of administration. * $p < 0.05$ vs. post-surgery, $n = 5-6$. B) All rats showed equivalent pre-conditioning time spent in the conditioning chambers. As no differences were observed between treatment groups, pre-conditioning values were pooled for graphical representation. Administration of the same dose of anti-NPY into the ipsilateral n. gracilis failed to alter time-spent in the conditioning chambers in sham or SNL treated rats, $n = 5-10$.