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Functional distinction between NGF-mediated plasticity and regeneration of nociceptive axons within the spinal cord

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Abstract

Successful regeneration after injury requires either the direct reformation of the circuit or the formation of a bridge circuit to provide partial functional return through a more indirect route. Presently, little is known about the specificity of how regenerating axons reconnect or reconstruct functional circuits. We have established an *in vivo* dorsal root entry zone model, which in the presence of NGF, shows very robust regeneration of peptidergic nociceptive axons, but not other sensory axons. Expression of NGF in normal, non-injured animals leads to robust sprouting of only the peptidergic nociceptive axons. Interestingly, NGF-induced sprouting of these axons leads to severe chronic pain, whereas, regeneration leads to protective like pain without chronic pain. Using this model we set out to compare differences in behavioral outcomes and circuit features between these two groups. In this study, we examined pre-synaptic and post-synaptic markers to evaluate the relationship between synaptic connections and behavioral responses. NGF-induced sprouting of CGRP axons resulted in a significant redistribution of synapses and cFos expression into the deeper dorsal horn. Regeneration of only the CGRP axons showed a general reduction in synapses and cFos expression within laminae I and II; however, inflammation of the hindpaw induced peripheral sensitization. This data shows that although NGF-induced sprouting of peptidergic axons induces robust chronic pain and cFos expression throughout the entire dorsal

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horn, regeneration of the same axons resulted in normal protective pain with a synaptic and cFos distribution similar, albeit significantly less than that shown by the sprouting of CGRP axons.

Keywords

Plasticity; Regeneration; Nerve growth factor; Hyperalgesia; Nociceptor

Introduction

The capacity of regenerating axons to reform original circuits is unknown, but this process could be influenced by numerous factors including: the regeneration of multiple axon populations, re-innervation of original pattern formation, formation of aberrant synapses, and competition for synaptic connectivity on projection and interneurons. In many systems these processes are difficult to analyze due to few axons regenerating the long distances required to reach targets, requirement of myelination for function and the complexity of the circuits. We have been examining the dorsal root entry zone model which provides clear evidence of neurotrophin-induced regeneration as well as sprouting. This model has the advantage of requiring axons to regenerate only a few millimeters into the spinal cord in order to establish functional connections involved in simple reflexes and pain transmission. This model also has the advantage of supporting robust regeneration of a single axon population (Ramer et al., 2000, Romero et al., 2001) or multiple axon populations (Wang et al., 2008) depending on the neurotrophins applied.

Our previous studies demonstrated that direct injection of adenovirus encoding NGF into the dorsal horn results in robust sprouting or regeneration of only peptidergic nociceptive axons (Romero et al., 2001; Tang et al., 2004; Tang et al., 2007). Although the general outgrowth and density of these calcitonin gene related peptide (CGRP) axons throughout the dorsal horn were very similar, we observed distinct behavioral differences between animals in which NGF was used to induce sprouting compared to those in which NGF was used to induce regeneration. In uninjured animals, the sprouting of CGRP-positive axons induced severe and persistent painful responses as shown by thermal hyperalgesia and mechanical allodynia (Romero et al., 2000; Tang et al., 2004). After dorsal root lesions, however, the regeneration of CGRP-positive axons, in the absence of all other sensory innervation, induced recovery of thermal nociceptive function close to normal baseline levels, showing normal-like protective pain without the progression into persistent, continual pain (Romero et al., 2001; Tang et al., 2007; Smith & Onifer, 2010).

Over the years, numerous papers have shown that NGF-produced from glia or immune cells after spinal cord injury induces sprouting of axons into the deeper dorsal lamina and the progression of chronic pain or autonomic dysreflexia (Lewin et al., 1994; Cameron et al., 2006; Christensen and Hulsebosch, 1997a, b; Krenz and Weaver, 2000; Gwak et al., 2003; Brown et al., 2004). Hyperinnervation of these axons onto neurons within the deeper dorsal horn was thought to be one of the primary mechanisms by which NGF leads to the establishment of chronic pain (Christensen and Hulsebosch 1997b; Romero et al., 2001; Brown and Weaver, 2012). Indeed, we previously observed that co-application of

Semaphorin 3A with NGF in uninjured rats was capable of reducing sprouting and mechanical hyperalgesia (Tang et al., 2004). Likewise, similar co-treatment after dorsal root rhizotomy leads to refined innervation and better targeting of regenerating axons to the upper dorsal horn; however, these animals showed no overt change in pain responses when compared to injured rats co-treated with NGF and GFP (Tang et al., 2007). These previous studies indicate that NGF induced hyperinnervation of central projection only partially describes a potential mechanism for induction of thermal and mechanical hyperalgesia, and other conditions, such as the growth state of the neurons and/or the involvement of other sensory pathways, may provide a secondary requirement for progression to a chronic pain state. We hypothesize that regeneration of only the peptidergic population of sensory afferents is insufficient to drive neuropathic responses, but is useful in reestablishing partial recovery of normal protective-like pain responses.

Materials and Methods

Animals

A total of sixty three adult (225–250g) female Sprague Dawley rats (Harlan Sprague Dawley, Indianapolis, IN, USA) were used in this study. All surgical procedures and animal maintenance complied with the NIH guideline regarding the care and use of experimental animals and were approved by the Institutional Animal Care and Research Advisory Committee. All studies with the exception of figure 3B follow the descriptions and end points described in Figure 1.

Adenoviral vectors

Replication-deficient recombinant adenovirus expressing nerve growth factor was constructed as previously described (Romero et al., 2000; Tang et al., 2004 and Tang et al., 2007). Briefly, plaque-purified recombinant adenovirus was propagated in HEK293 cells and purified by double cesium chloride gradient ultracentrifugation. Lack of wild type contamination was confirmed by PCR against both the E1A region (wild-type specific) and the E2B region as an internal control (Zhang et al., 1995). The number of viral particles (vp) was measured by optical absorbency, while the number of infectious particles (pfu) was determined using the Adeno-X Rapid titer kit (BD Bioscience/Clontech, Palo Alto, CA). The vp/μl (2.4×10^7) to pfu/μl (7.6×10^6) ratio was ~3:1. The expression of NGF following adenoviral infection of cells in culture was confirmed by western blot as described previously (Tang, et al., 2007).

Dorsal Root Crush Injury

Animals were deeply anesthetized by i.p. injections of a 2:1 mixture of ketamine (67mg/kg) and xylazine (6.7mg/kg). The back of each animal was clipped and disinfected with povidone-iodine before opening the skin and fascia. A hemilaminectomy (n=32) was performed under aseptic conditions at the L1-L2 vertebral segments and the dura mater opened with fine scissors. After topical application of lidocaine, dorsal roots L3-L6 were identified and exposed. With the use of #5 Dumont forceps, triple-crush lesions of 10 seconds were inflicted at two sites separated by 3mm along the L4 and L5 afferents at 5–8 mm from the DREZ. Dorsal roots L3 and L6 were tightly ligated at two regions, 1–2mm

apart with 6.0 silk suture and then a complete cut was made through these roots to create a zone of denervation immediately rostral and caudal L4/L5. All lesions were performed unilaterally on the right side. The spinal cord was covered with gelfoam soaked in sterile saline and the muscle and skin layers were closed together with chromic gut suture and surgical clips, respectively. Picric acid was applied to the right paw to help prevent autophagia, which sometimes occurred (~20%). Any animals showing loss of more than two toes or hindlimb inflammation were immediately euthanized and removed from the study. The animals were maintained on a heated pad until full recovery from anesthesia. Analgesic in the form of children's liquid acetaminophen (0.5mg/ml) was applied to the drinking water for two days following surgery to help alleviate pain and discomfort.

Adenoviral Injection into the Spinal Cord

Naïve animals or those that had undergone dorsal root crush two weeks previously, were anesthetized as described above and a hemilaminectomy was performed at vertebral levels T13-L1. The lumbar spinal cord was exposed and the dura mater was left intact. Eight injections of 0.3µl adenovirus (2×10^5 pfu/µl diluted in sterile saline) spaced 0.5 mm apart rostro-caudally along the DREZ at L4-L5 spinal segments, were made using a beveled glass micropipette pulled to a diameter of between 30 and 50 µm. Injections were made at a rate of 5 nl/sec using a nano-injector (World Precision Instruments, Inc., Sarasota, FL) at a precise depth of 0.7mm from the spinal cord dorsal surface using the coordinates on a M3301 fine micromanipulator (Narishige via World Precision Instruments). After completion of each injection, the needle remained in place for five minutes before retracting. Immediately prior and the day following virus administration, all animals received 100µg of 1:1 mixture of CD-4 (W3/25) and CD-45 (MRC OX-22) antisera to transiently suppress the immune response (Romero and Smith, 1998).

Fos Stimulation

One day after the final behavioral assessment (time points as described in figure 1), 3–5 randomly selected animals per group were anesthetized with 50mg/kg sodium pentobarbital i.p., a dose known to block flexor-reflex responses to hindpaw stimulation ~10 min after injection (Trafton et al., 1999). For Fos induction by thermal stimulation, both hindpaws were simultaneously submerged in water heated to 52°C for 2 min. The animals were placed on a heating pad for 90 min before perfusion.

Immunohistochemistry

Following the time points describe in figure 1, animals were anesthetized with sodium pentobarbital (Nembutal, 0.2ml/100g), perfused transcardially with 0.9% saline, followed by 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.5. Lumbar spinal cord segments were dissected and incubated in the same fixative for 24 hours and then in a 30% sucrose solution for 2–4 days at 4°C. Spinal cord tissue blocks were placed into a mold containing OCT and immediately frozen on dry ice. Thirty-micron floating sections were cut on a cryostat. Before staining all sections were treated with 5% H₂O₂ in 100% methanol to remove all endogenous peroxidase activity (e.g., red blood cells) and non-specific antibody blockade using 10% normal goat serum (goat secondary) or 10% donkey serum (donkey secondary antibody) in PBS with 0.3% Triton X-100. For immunoperoxidase staining, spinal cord

sections were incubated with rabbit polyclonal antiserum against rat calcitonin gene related peptide (CGRP; 1:20,000; Sigma, St. Louis, MO, C8198) or goat anti-human cFos (1:100; Santa Cruz Biotech. SC-52G). Visualization was achieved by incubating tissue in biotin-conjugated goat anti-rabbit (1:2000, Jackson ImmunoResearch, 111-065-003) or biotin-conjugated donkey anti-goat (1:2000, Jackson ImmunoResearch, 705-065-003) secondary antibodies followed by the Vectastain Elite ABC reagents (Vector Laboratories, Burlingame, CA), and developed with the peroxidase substrate, 3,3-diaminobenzidine to generate a brown color. All Vectastain procedures were done using concentrations and methods described in manufacture protocol. For double fluorescence staining, spinal cord sections were simultaneously incubated in anti-CGRP (1:1000) and mouse anti-rat synaptophysin (1:400; Sigma, S5768). Red and blue fluorescence labeling was achieved by additional incubation in goat-anti rabbit Texas Red (1:600, Jackson ImmunoResearch) together with a goat-anti-mouse biotinylated secondary antibody (1:600, Jackson ImmunoResearch), followed by incubation with AMCA-streptavidin (1:100, Jackson ImmunoResearch) for 3 hours. After extensive washing, sections were coverslipped with 5% propyl-gallate in glycerol for confocal microscopy.

Confocal Microscopy

Synaptic-like profiles were quantified as described previously (Tang et al., 2007) from 3 randomly selected section/animal (n=5 per group) separated by at least 100 μm with the L4/L5 spinal cord region. Single optical sections through the dorsal horn were obtained with a Leica (Nussloch, Germany) TCS SP laser scanning inverted confocal microscope equipped with a 100x oil objective, argon/krypton, helium/neon, and multiphoton lasers. Fluorescent images were acquired in the near red (tetramethylrhodamine isothiocyanate, 600–680 nm) and UV (410–470 nm) range. Sequential scanning was performed with the resolution set to 1024×1024 pixels, and single optical sections $\sim 1 \mu\text{m}$ thick were captured. All focal planes containing labeled CGRP fibers and synaptophysin puncta were stacked. The stacked sections obtained with each laser were overlaid to identify colocalization between the blue CGRP fibers and the red synaptophysin puncta, which appear purple. For each spinal cord section, the medial, middle and lateral regions of the superficial (lamina I/II and lamina III) and deep dorsal (lamina IV/V) horn were scanned. Brightness and contrast were adjusted in Photoshop 5.5 (Adobe Systems, San Jose, CA) for better clarity of the blue-colored staining. Data is reported as number of puncta count in the 100 μm box per section.

Image Analysis

CGRP or cFos labeled spinal cord sections (3 randomly selected sections/animal within L4/L5 region) were examined under a 4x or 10x objective of an E800 epifluorescent microscope (Nikon, Tokyo, Japan) and digital images were captured using Metamorph Imagine software. To quantify the number of Fos-IR cells, high magnification (120x) images were opened in Adobe Photoshop, cropped, magnified and converted to black and white to enhance clarity and then manually counted. To improve accuracy in counting, only labeled cells showing cFos nuclear staining were marked using the eraser tool as they were counted. The density of CGRP labeling in spinal cord sections was quantified using Axiovision 4.1 software (Zeiss, Inc.). A $100 \mu\text{m}^2$ box was placed over the dorsal horn in each image and the thresholded CGRP pixel density was measured within the confines of

the boxed area. Data was expressed as the percent threshold area over the total ($100 \mu\text{m}^2$) area. To quantify the number CGRP-synaptophysin puncta in the confocal image stacks, images were opened in Axiovision 4.1 software. Purple regions of complete red and blue overlap were identified, and the pixel area of each purple colored region was measured. CGRP-synaptophysin labeled puncta greater or equal to $1 \mu\text{m}^2$ were considered single synaptic clusters and counted. The numbers of synaptic-like puncta across the three dorsal horn regions (mid, medial and lateral) per lamina were averaged across three dorsal horn sections per animal and compared between groups.

Carrageen injections

Four weeks after lesioning and treatment, a solution of 1 mg carrageen in 100 μl of saline was injected into the right hindpaw, and 100 μl of only saline into the glabrous skin of the left hindpaw, following the method of Morris (2003). Six hours later noticeable swelling on the hindpaw was apparent and we proceeded to perform von Frey measurements for mechanical hyperalgesia using the Chaplin method (see below). Animals were re-examined 24 hours later after the inflammation had dissipated.

Thermal Nociception

The latency of paw withdrawal from a radiant heat source was used to measure the response time to noxious thermal stimuli, as described previously (Hargreaves et al., 1988). Baseline paw withdrawal latencies (PWL) for each hindpaw were determined prior to and then once a week following dorsal root crush or adenovirus injection. Briefly, the rats were placed in a clear plastic chamber with a glass floor and allowed to acclimate for 10 min. A halogen lamp beneath the glass surface is used to direct an intense light beam onto the plantar surface of the hindpaw. Thermal intensities of 50% of the maximum power output of the lamp were tested once a week for the entire duration of the experiment. This intensity was chosen based on preliminary studies to give baseline responses of 10 seconds in normal animals. Paw withdrawal latency was detected automatically by a photocell and taken as a behavioral index of the pain threshold. A score significantly lower than baseline represented hyperalgesia. A maximum exposure time of 22 sec was used to ensure that no tissue damage occurred to the paw. PWL measurements were taken in duplicate at 10 min intervals by individuals blinded to the treatment.

Mechanical Hyperalgesia

The paw pressure test was used to measure mechanical hyperalgesia, as described by Randall and Selitto (1957). Briefly, animals were restrained in a mitt such that the hindpaws could hang free. Each paw, in turn, was placed between the apparatus surface of an Ugo-Basil Analgesymeter and a plastic point. Increasing weight was applied to the point by means of an attached metal disc. Pressure to the paw was applied at thirty two grams per second. The end point was designated as vocalization or the pulling of the hindpaw from the apparatus, with a cutoff of 300 grams. Responses were recorded three times for both hindpaws.

von Frey

Tactile sensitivity was evaluated by measuring the nociceptive response to a cutaneous mechanical stimulus, using the von Frey method as described by Chaplan et al. (1994). Briefly, rats were placed in a clear plastic chamber on a wire mesh floor. After a 10 min habituation period, a series of eight von Frey filaments (log value 4.17–5.46; North Coast Medical, Morgan Hill, CA) were applied to the mid-plantar region of the hindpaws, following an up-and-down profile (Dixon, 1980; Chaplan et al., 1994). Stimuli for each paw were presented with steadily increasing pressure until the filament bent or the animal responded. After 6–8 seconds, the next filament was presented in the same fashion. The total number of stimuli applied to each paw ranged from 5 to 9, depending on different response patterns. The 50% withdrawal threshold was calculated according to the equation $10^{[X_f + k\delta]/10\,000}$, where X_f is the log value of the von Frey hair used, k is a tabular value for the pattern of responses (Chaplan et al., 1994) and δ equals the mean difference (in log units) between stimuli (0.184 for our studies). A minimum of four up-and-down trials was performed by individuals blinded to the treatment. Behavioral responses to mechanical stimuli were also performed 24 hours pre-and 6 and 24 hours post intraplantar injection of carrageenan.

Statistical analysis

Data from the image analysis of synaptophysin puncta, CGRP density and Fos-IR nuclei were analyzed by one-way ANOVA followed by Tukey-Kramer or Student Newman Keuls *post hoc* test to determine significant differences between groups. Paw withdrawal latencies (PWLs) to thermal stimuli were analyzed using a mixed model ANOVA with repeated measures using SAS v 9.13, followed by Bonferroni/Dunn *post hoc* test to determine significant differences between groups. Behavioral responses to mechanical stimuli before and following carrageenan injection were compared by paired T-test for each experimental group. Data represent the mean $\pm$ SD. P values below the 5% probability level were considered significant.

Results

The extent of ectopic growth is similar between regenerated or sprouted nociceptive axons

The vast majority of peptidergic nociceptive axons express CGRP and comprise a subset of unmyelinated C-fibers, which express TrkA receptors and developmentally require NGF for growth and survival. Centrally projecting CGRP+ fibers enter the spinal cord at the dorsal horn where they terminate in lamina I and outer lamina II (II_o), as shown in Figure 1A. Crush injury of the dorsal root proximal to the DRG causes degeneration of afferent sensory axons and the loss of CGRP staining in the superficial lamina of the dorsal horn ipsilateral to the injury, while the contralateral side remains unaffected. The low level of CGRP staining visible at the most dorsal edge of the spinal cord on the side of the injury represents small numbers of collateral fibers in Lissauer's tract (Figure 1B). NGF over-expression in the dorsal horn of an uninjured animal, results in robust sprouting of CGRP+ axons from the superficial lamina extending into deep ventral areas of the dorsal horn (Figure 1C). Similarly, NGF overexpression in the dorsal horn following dorsal root crush injury, results

in regeneration of CGRP+ axons through the DREZ and robust growth throughout the dorsal horn (Figure 1D). Notably, images of spinal cord sections stained for CGRP from normal animals injected with adenovirus encoding NGF (Normal/NGF; Figure 2A) or animals that had undergone crush injury prior to NGF adenovirus administration (Crush/NGF; Figure 2A) were virtually indistinguishable. The insignificant difference in the amount of CGRP labeling between Normal/NGF and Crush/NGF was confirmed by quantifying the percent area of the dorsal horn occupied by CGRP labeled fibers (Normal/NGF $52.2 \pm 6.7\%$; Figure 2B compared to Crush/NGF $44.6 \pm 1.5\%$). Surprisingly, despite the similarity in the magnitude of CGRP labeling and extensive localization of CGRP+ nociceptive axons between Normal/NGF and Crush/NGF animals, a disparity is observed in their behavioral response to noxious thermal stimulation.

Following a noxious thermal stimulus applied to the plantar region of the hindpaw, the Normal/NGF animals experience thermal hyperalgesia, while the Crush/NGF animals demonstrate a protective pain response to the same degree as the control animals, but fail to develop thermal hyperalgesia. This is demonstrated by a decrease in the PWL ratio (Normal/NGF 0.41 ± 0.1 compared to Crush/NGF 1.06 ± 0.16 or control 0.903 ± 0.076 ; p values, Figure 3A) of the right paw (ipsilateral to the crush and/or adenovirus injection) to the left (non-injured control) paw. These data illustrate that neither the numbers of CGRP+ nociceptive axons, nor the ectopic growth pattern throughout the dorsal horn was capable of causing hyperalgesia when NGF was used to induce sensory axon regeneration. Although not quantified about 30% of NGF/normal animals show a guarding like behavior in which they sit in a hunched position with their ipsilateral hindpaw raised off the floor of the cage (Romero et al., 2001). Likewise, transient allodynia was observed with the application of increasing pressure to the hindpaw of normal rats treated with NGF, but not, untreated normal rats, or Crush/NGF treated rats (Figure 3B). This further indicates that other sensory modalities associated with tactile responses are missing (Crush/NGF) or not hyperactivated (Normal) with increasing pressure to the hindpaw.

Nociceptive axons which either regenerated or sprouted form synaptic connections throughout the dorsal horn

Given the difference in the behavioral response to noxious thermal stimulation between the Normal/NGF and Crush/NGF animals, we were surprised to find no significant difference in the amount of CGRP labeling in the dorsal horn between these two groups of animals. Since behavioral responses are dependent on synaptic connections between nociceptive axons and central neurons, we compared the distribution of synaptic-like connections in the dorsal horn between groups. CGRP labeling was co-localized with synaptophysin (a presynaptic terminal marker protein) and dorsal horn sections were imaged by confocal microscopy (Figure 4A). The majority of synaptic puncta in control animals were localized to laminae I/II ($91 \pm 5.9\%$), depicting the normal innervation pattern (figure 4A & B). However, in the Crush/NGF and Normal/NGF animals approximately half ($48.7 \pm 8.9\%$ and $55.5 \pm 2.9\%$, respectively) of the total synaptic puncta were located in lamina I/II while the other half was localized to ectopic dorsal horn lamina. Although the percentage of synaptic connections in lamina I/II to the total number in the dorsal horn was not significantly different between Normal/NGF and Crush/NGF animals, the absolute number of synaptic puncta in Lamina

I/II in Normal/NGF (69 ± 13.3) was higher than those for Crush/NGF animals (32 ± 18.9 ; $p < 0.01$, Figure 4B). With respect to only the Normal/NGF and Crush/NGF groups, these data suggested that the differences in the behavioral outcome might not be directly related to changes in the innervation pattern of nociceptive axons but rather the number of synaptic connections in the superficial dorsal horn lamina where nociceptive fibers terminate. However, this is most likely not the case because control animals contained the highest number of synaptic puncta in the superficial lamina without developing spontaneous hyperalgesia.

Stimulation of either regenerated or sprouted axons induces cFos activation in postsynaptic dorsal horn neurons

To determine whether the CGRP-synaptophysin puncta corresponded to functional synaptic connections, the immediate early gene, cFos was activated by simultaneously submerging both hindpaws in 52°C water. This temperature provides a noxious heat source that results in the stimulation of nociceptive afferents and activation of cFos in postsynaptic cells located in the dorsal horn of lumbar spinal cord. cFos-containing dorsal horn cells were examined by immunohistochemistry and cFos immunoreactive (IR) nuclei are localized to superficial dorsal horn lamina in the normal animal on both sides (Figure 5A). Spinal cords after crush injury contained no cFos-IR cells ipsilateral to the injury, whereas the contralateral side contained many cFos-IR nuclei (Figure 5B). Spinal cord sections from Normal/NGF and Crush/NGF animals showed a widespread distribution of cFos-IR cells throughout the dorsal horn (Figure 5C,D). Note that the sections stained for cFos as shown in Figure 5A–D were taken from the same animals as the sections stained for CGRP as shown in Figure 2. A comparison between these two figures shows that cFos-IR cells are localized primarily in those areas containing CGRP+ axons. The numbers of cFos-IR cells per section were manually quantified and compared between groups (Figure 5E). In control animals, the majority of cFos IR cells were found in superficial dorsal horn lamina (58.8 ± 2.89) compared to deeper lamina (18.46 ± 3.06 ; $p < 0.05$). Spinal cords from animals in the other treatment groups showed insignificant differences in cFos IR cells between superficial and deeper dorsal horn lamina. In the superficial dorsal horn, Normal/NGF animals contained significantly higher numbers of cFos IR cells (77.6 ± 16.5) when compared to Crush/NGF animals (40.5 ± 5.26 , $p < 0.001$) or control ($p < 0.01$). Similarly, more cFos IR cells were found in deep dorsal horn laminae of Normal/NGF (102.1 ± 21.3) versus Crush/NGF animals (32.08 ± 15.26 , $p < 0.01$) or controls ($p < 0.01$). These data confirm that regenerated and sprouted nociceptive axons form functional synaptic connections throughout the dorsal horn, and the numbers of functional connections correlates with the development of thermal hyperalgesia.

Peripheral inflammation induces mechanical allodynia after regeneration of peptidergic nociceptive axons

To determine if regenerating axons maintained their “normal-like” peripheral responses, another cohort of animals for the 4 groups described in figure 1 were examined for peripheral sensitization. Mechanical allodynia was measured using von Frey hairs (up & down method, Chaplan et al. 1994). Pre-carrageen baselines indicated right/left paw ratios of 1 for normal and crushed/NGF treated groups (Fig. 6), whereas, non-injured animals treated

with NGF showed an allodynic response (ratio < 1.0). The crush control group showed no response throughout and they were not included in the figure. Six hours after carrageen injections, the right hindpaw was noticeably swollen, with only the control and Crush/NGF groups showed a statistically significant reduction in pain thresholds when compared to pre-carrageen (Fig 6), which returned to baseline levels the next day (student T-test * $p < 0.05$, ** $p < 0.01$, compared to pre-carr baselines). This data shows that regenerated peptidergic nociceptive axons maintain sensitization, without the initial neuropathic pain associated with NGF treatment of non-injured animals.

Discussion

In normal tissues of the spinal cord, endogenous NGF is at very low concentration and exogenous administration result in different behavioral responses depends on different injection routes and animal models (Lewin et al., 1993; Verge et al., 1995; Malcangio et al. 2000; Ramer et al., 2000; Cahill et al., 2003). Our study injected adenovirus encoding NGF into the dorsal horn of normal animals or after dorsal root crush injury. Results show robust sprouting and regenerating calcitonin gene related peptide (CGRP) throughout the dorsal horn. Although the area occupied by CGRP-axons between NGF normal and NGF crush were similar, different behavioral responses were found. Normal animals injected with NGF displayed extensive thermal and mechanical hyperalgesia; whereas, injured animals injected with NGF displayed no neuropathic pain responses and only demonstrated protective pain. In both treatment groups, synaptic connections and nociceptive induction of cFos were identified at normal locations (lamina I & II) as well as increased in abnormal regions within the deeper dorsal lamina.

Nerve growth factor is known to induce multiple transcriptional and morphological changes in adult sensory neurons, with one of the most profound being the extensive branching of their axons (Spillane et al., 2012). Beads soaked in NGF induce filopodial formation and the progressive development of axon branches at the location of contact (Gallo and Letourneau, 1998). Peripheral application or expression of NGF promotes extensive branching of cutaneous terminals within the skin (Davis et al., 1997) and hyperexcitability in DRG neurons (Radtke et al., 2010). Expression of NGF within the spinal cord induced branching of these fibers out of lamina I and II into the deeper dorsal laminae. Interestingly, the movement of axons out of lamina I and II also induced mobility of synaptic connections, in which there was a significant reduction in the number of synaptic-like profiles in the upper dorsal horn and a general increase in synapses within the deeper dorsal horn, with the total number of synapses between normal and normal/NGF treatment being similar. Although we observed this potential mobility for synapses stained with synaptophysin, a subpopulation of primary sensory axons express synaptoporin and not synaptophysin (Sun et al., 2006), the potential mobility of these terminals were not assessed. Branching factors, such as netrin-1, Slit-1, and BDNF are also known to regulate synaptic density, indicating these two processes could be linked (Campbell et al., 2007; Marshak et al., 2007; Manitt et al., 2009) and filopodial formation required for sprouting is suppressed at sites of mature synapses (Meyer and Smith, 2006). In the visual system, retinal ganglion cells show an inverse relationship between arbor size and synaptic density (Hornberg et al., 2013). This homeostatic mechanism is thought to stabilize visual neural circuits in response to

alterations in synaptic activity (Hornberg et al., 2013; Shah and Crair, 2008). Whether a similar mechanism exists within the somatosensory system is unclear; however, we have previously observed that co-expression of semaphorin 3A and NGF reduced nociceptive axon sprouting, mechanical hyperalgesia, and increased the synaptic density within lamina I and II (Tang et al., 2004; 2007), indicating the presence of a homeostatic mechanism to regulate synaptic density along afferent arbors.

In the mouse, peptidergic nociceptive axons are associated mostly with thermal nociception, whereas, mechanical nociception is mediated by non-peptidergic sensory afferents. Ablation of the TRPV1 subset of peptidergic neurons results in the loss of thermal nociception with little change in mechanical hyperalgesic responsiveness; however, ablation of essentially all of the IB4 (non-peptidergic) population selectively eliminated mechanical nociception, despite the polymodal nature of these neurons (Zhang et al., 2013). Regeneration exclusively of peptidergic nociceptive axons resulted in recovery of thermal nociception, but not mechanical nociception, further indicating this to be a property of the NGF responsive population of nociceptive axons. However, NGF-induced sprouting in the presence of the other sensory afferents resulted in both thermal and mechanical hyperalgesia, most likely due to convergence of these polymodal nociceptors onto wide dynamic range neurons within lamina V. Retraction of these terminals in the presence of Semaphorin-3A would account for the loss of mechanical hyperalgesia after NGF expression within the spinal cord (Tang et al., 2004). Eventhough regenerating peptidergic nociceptive axons grew into and synapsed within the deeper dorsal horn, the activation of these neurons failed to induce mechanical sensitivity, even to normal levels. Further demonstrating the importance of other sensory afferents in mediating this response. Interestingly, peripheral inflammation was capable of driving mechanical hyperalgesia through regenerated peptidergic nociceptive axons, indicating these polymodal nociceptive axons possess the capacity to drive mechanical sensitivity under specific pathological conditions.

We have previously shown that NGF expression within the normal cord induces severe persistent pain, which is absent with crush/NGF (Romero et al., 2001; Tang et al., 2004, 2007). Within normal/NGF treated groups, there was a distinct loss of synaptic-like profiles (46% of normal) in the upper dorsal horn, however, the number of cfos labeled cells within this region increased (132% of normal) when compared to cfos levels in untreated animals. Similar differences were observed after NGF induced regeneration of CGRP+ axons, in which cfos levels were 68% of normal, eventhough synaptic density was only 23% of normal. This difference indicates a potential strengthening of the excitatory drive in these neurons, that could be due to stronger more efficient synapses being formed or increased hyperexcitability of spinal interneurons associated with activated microglia within the dorsal horn (Woolf and Salter, 2000; Tsuda et al., 2003). Activated microglia were identified within the spinal cord in both treatment groups (not shown), but at a slightly qualitatively higher level after rhizotomy. Reactive microglia within the dorsal horn are known to increase hyperexcitability of dorsal horn projection neurons by inverting the polarity of currents induced by GABA (Coull et al., 2003; 2005). Synaptic release of GABA induced depolarization instead of hyperpolarization within the nociceptive projection neurons, thus amplifying afferent nociceptive signals, driving neuropathic pain responses. The lack of progression into neuropathic pain after crush injury and administration of NGF could be due

to the low levels of synapses established within the superficial dorsal horn. Under such a condition, the increased excitatory gain from the reversal of anionic potentials would be insufficient to drive neuropathic responses. On the other hand, the Gate Control Theory of pain proposes co-activation of Ab afferents suppress nociceptive outputs by activating inhibitory interneurons and reducing excitatory neurotransmitter release from nociceptive presynaptic terminals (Melzack and Wall, 1965). Increase in Cl⁻ levels due to loss of cation-chlorine-cotransporters could increase Cl⁻ efflux from the pre-synaptic nerve terminals converting non-noxious Ab stimulation into noxious mechanical allodynia (Price et al., 2005). This excitatory drive would be absent with NGF-induced regeneration since Ab fibers do not regenerate with that treatment.

The mobility of afferent terminals into abnormal dorsal horn regions indicates a profound alteration in connectivity within the dorsal horn (Tan and Waxman, 2012). This process occurs to a similar percentage of total synapses in both NGF treatment groups, irrespective of injury or the presence of other sensory axons. The difference in synaptic-like profiles and cfos was most profound within the deeper dorsal lamina of the normal spinal cord expressing NGF. Within the deeper dorsal laminae, synaptic-densities increased 3.9 fold for normal/NGF and 2.9 fold for crush/NGF when compared to the untreated group; whereas, cfos increased 5.7 fold for normal/NGF, but only 1.7 for crush/NGF. Since cfos does not provide a direct measure of excitation of individual afferent populations but represent a more global measure of neuronal excitability, the dramatic increase in cfos for normal/NGF could also be partially induced by activation of other sensory afferents or non-peptidergic nociceptive axons, which are absent in the crush/NGF group. Typically, peptidergic nociceptive afferents primarily terminate directly on lamina I interneurons and projection neurons, and a few of these afferents and the non-peptidergic (IB4) afferents indirectly activate lamina V projection neurons (Braz et al., 2005; Braz and Basbaum, 2009). This establishes two parallel pain pathways, with convergence onto hypothalamic and amygdala targets, but divergent onto thalamic and globus pallidus. The latter of which is associated with motor and postural abnormalities associated with neuropathic pain (Braz et al., 2005). The migration of axonal sprouts and synapses into the deeper dorsal horn lamina suggest a reduced synaptic input onto lamina I projection neurons and an increase in activation of lamina V wide-dynamic range neurons by peptidergic nociceptive afferents. This may partially explain the increase in mechanical hyperalgesia, allodynia, and the hindpaw guarding behavior associated with NGF induced sprouting in non-injured animals. On the other hand, regeneration of only the peptidergic population and absence of regeneration for non-peptidergic nociceptive and Ab axons does not provide sufficient drive to elicit tactile allodynia in the absence of peripheral inflammation. Growth of these axons into the deep dorsal horn could bypass the theorized flow of pain information from the superficial laminae into the deeper laminae (Wall 1960; Ritz and Greenspan, 1985; Braz and Basbaum, 2009) driving mechanical nociception by peptidergic afferents polymodal nociceptors.

These data are significant because, individually, overexpression of NGF (Krenz et al., 2000; Romero et al., 2000), spinal nerve lesions (Kim and Chung, 1992), or spinal cord injury (Christensen and Hulsebosch, 1997) have a high probability of inducing central sensitization and the progression to neuropathic pain; however, NGF-induced regeneration of only the peptidergic nociceptive pathway in the absence of other afferent sensory input produces only

acute, protective pain responses. Although neuropathic pain is a pathological state, it is the normal manifestation of central sensitization and modulation of the endogenous pain pathways. The ability of regenerating peptidergic nociceptive axons to induce protective pain, and to respond to sensitization shows that they have established functional circuits. The inability of these regenerating axons to induce the pathological state of neuropathic pain strongly indicates that they are missing an essential component(s) of the endogenous circuit. The lack of regeneration of other sensory modalities is most likely one of the important missing components. However, recovery of mechanical hyperalgesia with peripheral inflammation suggest the growth of these polymodal afferents into the deeper dorsal horn adapt to participate in mechanical nociception.

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The paper explores functional differences between regenerating and sprouting nociceptive fibers.

NGF-mediated sprouting of CGRP axons induces hyperalgesia.

NGF-mediated regeneration of only CGRP axons after rhizotomy induces protective pain.

Peripheral inflammation can induce tactile hyperalgesia after NGF-mediated regeneration.

Synaptic numbers, cFos expression and localization are different between the 2 treatments.

Figure 1.

Experimental paradigm and staining pattern of CGRP+ nociceptive afferents. **A**, L4/L5 rat spinal cord sections examined by immunohistochemistry illustrated the normal innervation pattern of termination of CGRP-containing sensory axons in lamina I and outer lamina II. **B,D**, Rats underwent a double-crush injury on right L4, L5 dorsal roots, and the adjacent L3, L6 dorsal roots were cut to prevent collateral sprouting from these segments (the left side shows sham control). After dorsal root injury, CGRP+ axons were absent from this region except for a small number of collateral fibers in Lissauer's tract as seen in **(B)**. Loss of CGRP staining in L3-L6 dorsal horn occurred within a few days and remained even at six weeks, demonstrating that no spontaneous regeneration into the spinal cord occurred. **C**, NGF overexpression in the dorsal horn of naïve animals that had not undergone crush injury, resulted in robust sprouting of CGRP+ axons throughout all dorsal horn laminae. **D**, Two weeks after crush injury, injections of adenovirus encoding NGF (NGF/Ad) were made ipsilateral to the side of injury (shown in blue). NGF overexpression in dorsal horn induced extensive regeneration of CGRP+ axons into the injection area, which occupied all dorsal horn laminae.

A) Withdrawal Latency (Thermal)

B) Withdrawal Latency (Pressure)

Figure 2.

NGF overexpression in the dorsal horn following crush injury or no injury induced robust regeneration or sprouting of CGRP+ axons, respectively. **A**, Magnified images of the dorsal horn stained for CGRP are shown for each treatment group (upper panel). Dorsal root crush injury virtually abolished CGRP+ fibers in the dorsal horn, while NGF overexpression induced growth and regeneration of CGRP+ fibers in the NGF crush group and robust sprouting of CGRP+ axons in the NGF normal group. Note the similarity between the NGF crush and NGF normal images in the magnitude of CGRP labeling. To determine the extent of growth of CGRP-containing axons per section, the area of the dorsal horn occupied by CGRP+ axons was measured in each group (**B**; lower panel). The difference in CGRP axon-occupying area between NGF normal and NGF crush was insignificant ($p > 0.05$, Tukey posthoc test). $**p < 0.01$, $*p < 0.05$ compared to normal.

Figure 3.

Behavioral response to treatment with nerve growth factor. Nociceptive responses were evaluated by measuring the PWL to thermal stimuli (A, n=8–9/group) or mechanical response to paw compression (B, n=3/group). Measurements were taken from the left (contralateral control) hindpaw and the right (experimental) paw at –3 days and one week following crush injury for the crush and Crush/NGF groups, as well as one to three weeks after injection of NGF/Ad into the Normal/NGF and Crush/NGF animals. Dorsal root crush injury caused a complete loss of paw withdrawal responses, raising the ipsilateral PWL to cutoff value (Crush/NGF and Crush groups compared to normal animals). NGF/Ad-induced regeneration (Crush/NGF group) results in recovery of thermal nociception and decreased PWL to near-normal levels (A); however, NGF did not increase withdrawal response to applied pressure to the paw (B). NGF/Ad injection in normal animals (Normal/NGF) resulted in reduced PWL responses for both thermal nociception and pressure. Values represent mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$ when compared to non-injured controls.

Figure 4.

Confocal microscopy images of synaptic puncta associated with CGRP-containing axons in the dorsal horn. The spinal cord was stained for CGRP (blue) and synaptophysin (red) and synaptic clusters (purple) were counted. **A**, CGRP-synaptophysin puncta are localized to superficial laminae in the dorsal horn of normal animals, with very few appearing in deeper laminae (left top panel, scale bar, 100 μ m). Higher magnification images of laminae I and II (superficial dorsal horn) and lamina III–V (deep dorsal horn) confirm the synaptic pattern in normal animals (left middle and lower panel; scale bar 10 μ m). In contrast, a high density of CGRP-synaptophysin clusters is present in all laminae of the dorsal horn in NGF crush and NGF normal animals (middle and right panel). **B**, Graph showing the distribution of synaptic puncta within laminae I/II, III and IV–V for each treatment group. The widespread distribution of synaptic puncta within the dorsal horn of NGF normal and NGF crush groups is in stark contrast to normal animals, where the majority of synaptic clusters are confined to laminae I and II. Despite the similarity in synaptic profiles, the numbers of synaptic puncta

present in lamina I/II in NGF normal animals is higher than that found in NGF crush animals. Values represent mean $\pm$ SD. Tukey posthoc test. * $p < 0.05$, ** $p < 0.01$ compared to Normal distribution, # $p < 0.01$ comparison of Normal/NGF to Crush/NGF.

Figure 5.

Fos expression in the dorsal horn as a measure of postsynaptic activation by regenerated and sprouted CGRP axons. Ninety minutes before animals were perfused, both hindpaws were simultaneously submerged in water warmed to 52°C, a temperature known to produce a noxious thermal stimulus. The pattern of Fos activation was determined by immunohistochemistry. **A**, Fos-IR cells are present in laminae I and II of the dorsal horn in normal animals, with very few in the deeper laminae. **B**, Following dorsal root crush injury a dramatic loss of Fos-IR cells is found on the ipsilateral side, while the contralateral side remains unaffected. **C**, High numbers of cFos positive cells are localized throughout the ipsilateral NGF expressing dorsal horn (right side), with normal distribution on contralateral side (left side). **D**, Regeneration of CGRP+ axons into the dorsal horn leads to Fos activation in cells throughout the dorsal horn, which is similar to the distribution pattern present in the dorsal horn of NGF normal animals. **E**, Graph showing the distribution of Fos IR cells in the superficial and deep dorsal horn laminae between groups. As seen in the images, the majority of Fos-IR cells were present in the superficial laminae in normal animals, while in NGF crush and NGF normal animal Fos-IR cells were present in all dorsal horn laminae of equal proportion. However, higher numbers of Fos-IR cells were found in both superficial and deep dorsal horn laminae of NGF normal animals compared to NGF crush animals. Values represent $\pm$ SD, * p <0.05, Tukey test).

Mechanical allodynia after Carrageenan

Figure 6.

Peripheral administration of carrageenan induces mechanical allodynia in all treatment groups. Prior to treatment with carrageenan (pre-carr), non-injured animals injected with NGF/Ad showed a significant reduction in the Von Frey 50% threshold ratios when compared to the other two groups. Six hours after carrageenan treatment (6 hrs post-carr) all animals showed significant allodynia, even normal animals treated with NGF showed a further reduction in threshold response. Twenty four hours after hindpaw injection of carrageenan (24 hrs post-carr), threshold response returned to pre-carrageenan levels. In which NGF treated normal animals still showed significant persistent mechanical allodynia. Acute mechanical allodynia was observed 6 hours after injection of carrageenan into the ipsilateral hindpaw only in sham controls and after regeneration of CGRP-positive axons. Values represent mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, compared to pre-carr. # $p < 0.01$, compared to Normal or Crush/NGF.

Table 1

Animals numbers for each control or experimental group

Groups	Behavior/immuno	cFos expression	inflammation
Normal	N=12/n= 7	N=5	N=4
Crush	N=11/n= 7	N=3	N=4
Normal/NGF	N=12/n= 7	N=5	N=4
Crush/NGF	N=12/n= 7	N=5	N=4