



Rat dorsal horn neurons primed by stress develop a long-lasting manifest sensitization after a short-lasting nociceptive low back input

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Abstract

Background: A single injection of nerve growth factor (NGF) into a low back muscle induces a latent sensitization of rat dorsal horn neurons (DHNs) that primes for a manifest sensitization by a subsequent second NGF injection. Repeated restraint stress also causes a latent DHN sensitization.

Objective: In this study, we investigated whether repeated restraint stress followed by a single NGF injection causes a manifest sensitization of DHNs.

Methods: Rats were stressed repeatedly in a narrow plastic restrainer (1 hour on 12 consecutive days). Control animals were handled but not restrained. Two days after stress paradigm, behavioral tests and electrophysiological in vivo recordings from single DHNs were performed. Mild nociceptive low back input was induced by a single NGF injection into the lumbar multifidus muscle just before the recording started.

Results: Restraint stress slightly lowered the low back pressure pain threshold (Cohen $d = 0.83$). Subsequent NGF injection increased the proportion of neurons responsive to deep low back input (control + NGF: 14%, stress + NGF: 39%; $P = 0.041$), mostly for neurons with input from outside the low back (7% vs 26%; $P = 0.081$). There was an increased proportion of neurons with resting activity (28% vs 55%; $P = 0.039$), especially in neurons having deep input (0% vs 26%; $P = 0.004$).

Conclusions: The results indicate that stress followed by a short-lasting nociceptive input causes manifest sensitization of DHNs to deep input, mainly from tissue outside the low back associated with an increased resting activity. These findings on neuronal mechanisms in our rodent model suggest how stress might predispose to radiating pain in patients.

Keywords: Myofascial low back pain, Latent and manifest sensitization, Restraint stress, Referred low back pain, Nerve growth factor

1. Introduction

Nonspecific low back pain (LBP) is a common complaint and one of the leading causes of disability in patients with pain.^{26,44} Nonspecific LBP often radiates into the proximal lower limbs called pseudoradicular in contrast to radicular neuropathic pain induced by a disk prolapse.¹³ Low back pain may also be a part of the clinical presentation of chronic

widespread pain (CWP) and fibromyalgia.¹⁴ Stress is linked to the unfolding of clinical psychopathologies such as anxiety disorders and depression.^{2,8,9} Psychological trauma in humans is a risk factor of the chronicity of subacute LBP and the development of CWP.^{36,52}

Nerve growth factor (NGF) is associated with painful muscle disorders. Muscles are one of the sources of NGF,¹ and its production is higher in overloaded or injured muscles.^{16,37,59} Intramuscular NGF injections in humans do not elicit immediate pain sensations⁶ but induce long-lasting hyperalgesia.^{49,57} A recent study demonstrated that inhibiting NGF alleviated chronic LBP in patients.³³ Animal studies showed that intramuscular NGF injections activated nociceptive muscle afferents^{23,37} and sensitized dorsal horn neurons (DHNs) through subthreshold postsynaptic potentials and low-frequency activation.²⁴ A single NGF injection into a low back muscle induced a state of latent sensitization in DHNs, ie, the neurons behaved normally but were easier to sensitize by a subsequent nociceptive input. The resulting manifest sensitization was characterized by signs of mechanical hyperalgesia, increased resting activity, and receptive field (RF) expansions towards the hind limb to deep tissue stimulation.²² In rats, repeated stress did not induce behavioral signs of mechanical hyperalgesia but led to an increased resting activity of DHNs and a nonsignificant increase in responsiveness to deep tissue input.²⁵ In

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mice, the combination of stress and intramuscular NGF injections caused manifest hyperalgesia.^{31,32}

Thus, prolonged stress in animals may lead to a latent spinal sensitization, depending on the stress model and stress intensity. Stress- and NGF-induced mild nociceptive input may synergize in enhancing the responsiveness of DHNs, and this could explain why stress is a risk factor of chronic pain in humans.⁵²

In this study, we hypothesized that repeated restraint stress induces a latent sensitization of DHNs, and therefore, a subsequent mild nociceptive input should lead to a manifest sensitization of these neurons, affecting the sensitivity of the DHNs to deep tissue input from inside and outside the low back and increasing their resting activity. In other words, stress followed by 1 NGF injection was predicted to have similar effects as 2 repeated NGF injections with an interval of 5 days.²²

2. Materials and methods

2.1. Animals and treatment groups

The experiments were performed on 11 adult male Sprague-Dawley rats (body weight 380–450 g; control + NGF, $n = 6$; stress + NGF, $n = 5$). The experimental procedure was approved by the local ethics authority responsible for animal experimentation (Regierungspräsidium Karlsruhe, Germany) and was performed following the German law on the protection of animals and ethical proposals of the International Association for the Study of Pain.

2.1.1. Treatment groups with a single nerve growth factor injection

Data collection of the present investigation is presented in the Results section. All animals received a single injection of NGF into the multifidus (MF) muscle directly before the recording of the DHNs started (refer section 2.3; **Fig. 1**).

2.1.1.1. Stress + nerve growth factor

In 5 animals, repeated restraint stress was induced on 12 consecutive days for 1 hour every day in a narrow plastic tube (refer section 2.2).

2.1.1.2. Control + nerve growth factor

Six animals served as a control. The animals were handled on 12 consecutive days (transported to the laboratory and picked up by hand) similar to the stress animals but not by repeated restraint stress.

2.1.2. Treatment groups without additional nerve growth factor injection

Previously published data from²⁵ are shown for reasons of comparison in the figures, and besides, a reanalysis of resting activity stratified by location of RFs was performed.

2.1.2.1. Control

Six rats were handled similar to stressed animals but not repeatedly restrained.

2.1.2.2. Stress

Repeated restraint stress was induced on 12 consecutive days for 1 hour every day in 6 rats.

2.2. Repeated restraint stress

All animals were habituated for 1 week to laboratory conditions. Restraint stress was induced similar to previous studies^{15,25} by placing animals for 1 hour daily on 12 consecutive days in a narrow restrainer (inner length 17.5 cm; inner height 5.5 cm, **Fig. 1**). Body weight was measured on day 14 before anesthetizing the animal. There was a nonsignificant trend towards a lowered body weight in stressed animals (control + NGF: 437 ± 29 g; stress + NGF: 407 ± 21 g; $P = 0.052$). Signs of distress such as vocalization, struggling during restraint (escape movements), and/or defecation were observed during the stress paradigm.

2.3. Injection of nerve growth factor

Under final anesthesia, (refer to section 2.5), directly before the recordings started, 50 μ L of NGF at a concentration of 0.8 μ M (NGF, human recombinant; Calbiochem, Merck, Germany) dissolved in phosphate-buffered saline (pH of the NGF solution: 7.2–7.3) was injected into the left MF muscle at 3 mm lateral to the spinous process of L5.^{22,62} The NGF concentration used is known to induce hyperalgesia when intramuscularly injected into animals or humans.^{6,22,24,49}

2.4. Pressure pain threshold

To test for mechanical hyperalgesia or hypoalgesia caused by restraint stress, the pressure pain threshold (PPT) of the low back was determined before the following NGF injection with an electronic von Frey anaesthesiometer (Life Science Instruments, Woodland Hills, CA)

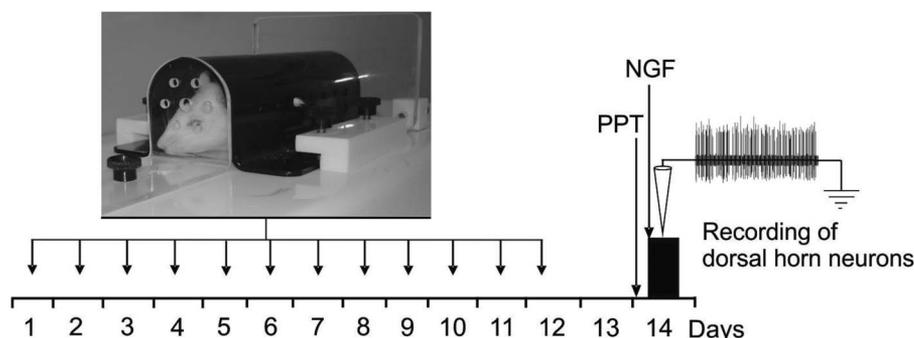


Figure 1. Experimental procedure. Animals of the stress group were repeatedly stressed in a narrow plastic restrainer on 12 consecutive days for 1 hour every day. The pressure pain threshold (PPT) of the multifidus muscle at vertebral level L5 was measured on day 14. On the same day, recordings of single spinal dorsal horn neurons were made (black bar). All animals received a single injection of NGF into the multifidus muscle directly before the electrophysiological recordings but after the PPT measurement. NGF, nerve growth factor.

on day 14 (Fig. 1). The blunt tip with an area of 3.46 mm² was pressed with increasing intensity to the MF muscle through the intact skin at the vertebral level L5. With the blunt tip, mainly nociceptors in deep tissues but not in the skin were excited.⁵¹ The PPT was defined as the minimum of pressure intensity required to elicit a pain-related reaction (withdrawal and escape movements and vocalization).

2.5. Recording of spinal dorsal horn neurons

2.5.1. Surgical procedures

In the final electrophysiological experiment, the animals were deeply anesthetized with thiopental sodium (Trapanal; Atlanta Pharma, Germany), 100 mg/kg intraperitoneally initially, followed by constant intravenous (i.v.) infusion into the external jugular vein (of the same anesthetic) at a rate of 10 to 20 mg/kg/h using an infusion pump to maintain a deep and constant level of anesthesia abolishing flexor reflexes or blood pressure reactions exceeding 10 mm Hg to noxious stimuli. Muscular relaxation was induced with pancuronium bromide (Inresa, Germany; 0.5 mg/kg i.v. initially and later 0.3 mg/kg/h i.v.). The mean arterial blood pressure was measured in the right common carotid artery, and body core temperature was continuously monitored and maintained at physiological levels (above 80 mm Hg, 37–38°C). The animals were artificially ventilated through a tracheal cannula with a gas mixture of 47.5% O₂, 2.5% CO₂, and 50% N₂.

A laminectomy was performed to expose the lumbar spinal segments L1 to L5. The dorsal roots L3 to L5 were exposed for applying the electrical search stimulus. The laminectomy did not affect the caudal back muscles and the overlying fascia at vertebral level L5 because the spinal segments were located 3 to 5 cm cranially from these muscles.⁵⁰ A pneumothorax was performed to reduce respiratory-related movements. The animals were euthanized at the end of experiments under deep anesthesia with an overdose of the anesthetic.²²

2.5.2. Recording of single dorsal horn neurons

Extracellular recordings from single DHNs were made in the spinal segment L2 ipsilateral to the site of NGF injection. Segment L2 receives strong input from deep tissues in the low back located at vertebral level L5,⁵⁰ and much of this input is directed to deep dorsal horn.^{5,35} Recordings were made with glass microelectrodes filled with 5% NaCl. Microelectrode penetrations were made to a depth of 1000 μm. For an unbiased sampling of DHNs, an electrical search stimulus of the dorsal roots L3, L4, and L5 at A-fiber strength was used during microelectrode tracking (intensity 5 V, width 0.3 ms, and repetition rate 0.33 Hz).⁵⁰ All DHNs exhibiting a stable response to this search stimulus were accepted for the study. A search stimulus at C-fiber strength was not used to avoid long-term potentiation.⁶³

2.6. Identification of receptive fields and neuron classification

The search for RFs of each neuron was performed following a standardized protocol in both experimental groups as previously described.²⁵ The RFs were identified by mechanical stimulation of low back structures, the left hind limb, hip, lateral abdominal wall, and tail. The responsiveness to mechanical stimulation on the right body side was not tested because previous studies had not shown any evidence for contralateral RF expansions in our model.^{22,25}

The types of mechanical stimulation used were as follows: as an innocuous stimulus touch with an artist's brush; as noxious stimuli, pinching with a sharpened Watchmaker's forceps (skin and thoracolumbar fascia); or as noxious pressure with a blunt probe (muscle). The deep RFs outside the low back were identified using a blunt probe. Deep tissues of the low back such as thoracolumbar fascia and muscle could be tested directly because the overlying skin was removed (refer to section 2.5.1; laminectomy).

Table 1

Input sources of dorsal horn neurons recorded in this study.

Groups	Control + NGF	Stress + NGF	P
Total number of neurons	29	31	
Recording depth	650 (745–460)	510 (720–380)	
Latency	1.8 (2.1–1.6)	1.8 (2.1–1.8)	
Neurons with deep input	4/29 (14%)	12/31 (39%)	0.041
Recording depth	750 (798–568)	620 (803–403)	
Low back	2/29 (7%)	4/31 (13%)	0.671
Outside low back	2/29 (7%)	8/31 (26%)	0.081
Neurons with skin input	19/29 (65%)	19/31 (61%)	0.793
Recording depth	660 (710–420)	590 (770–380)	
Neurons with convergent input	2/29 (7%)	7/31 (23%)	0.147
Recording depth	755 (800–710)	640 (830–470)	
Neurons without receptive fields (RFs)	8/29 (28%)	7/31 (23%)	0.768
Recording depth	620 (850–453)	470 (510–305)	
Neurons with resting activity	8/29 (28%)	17/31 (55%)	0.039
Recording depth	685 (725–520)	510 (685–345)	
Resting activity, with deep input	0/29 (0%)	8/31 (26%)	0.004
Resting activity, with skin input	8/29 (28%)	12/31 (39%)	0.419
Resting activity, with convergent input	0/29 (0%)	5/31 (16%)	0.052
Resting activity, without receptive fields	0/29 (0%)	2/31 (6%)	0.492

Recording depth and latency are expressed as the interquartile range: median (quartile 3–quartile 1).

The proportion of neurons is expressed as the number of neurons recorded to a specific input type/total number of neurons recorded for a group (percentage of the response). *P* values are calculated using Fisher exact probability test, and *P* < 0.05 was considered significant.

The table gives the number of neurons recorded for each group studied. Control + NGF: animals that were handled but not stressed and received single NGF injections into the multifidus muscle. Stress + NGF: animals that were stressed by repeated restraint and subsequently received single NGF injections. Recording depth and latency are shown for total neurons recorded and recording depth for each input type. Deep tissues: neurons with input from deep tissues (eg, muscle and fascia); skin: neurons with input from the skin; convergent: neurons with input from deep tissues and skin. Without receptive fields: neurons that responded to the electrical search stimulus but could not be excited with the mechanical stimuli used. Resting activity: neurons that show spontaneous activity without presenting any mechanical or chemical stimuli.

NGF, nerve growth factor.

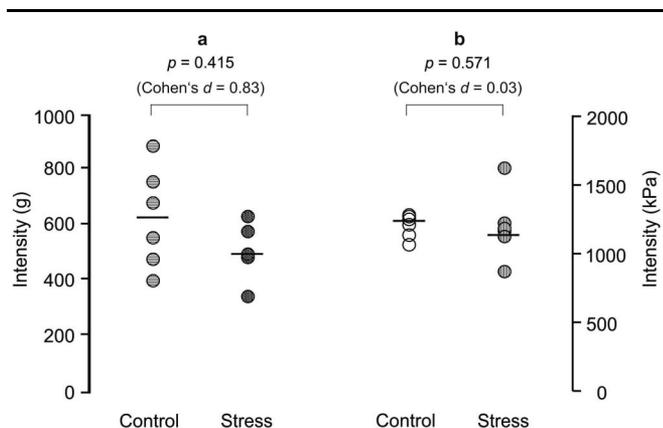


Figure 2. Pressure pain threshold of the low back before NGF injection. The force (in “g” on the left y-axis) required to elicit a pain-related reaction (withdrawal behavior, escape movements, and vocalization) using a blunt probe with an area of 3.46 sq. mm when applied to the multifidus muscle of the low back. For comparison with human data, the right y-axis shows pressures in “kPa.”⁴⁴ (A) Data of this study; note that thresholds were measured before the NGF injection (Fig. 1). (B) Data of a previous study with a similar stress paradigm but without NGF injections.²⁵ Black horizontal lines indicate the median for each group. NGF, nerve growth factor.

A neuron responding to touching or pinching of the skin was considered as having cutaneous input. Neurons responding to pressure applied to a muscle or other deep tissues with a blunt probe were considered having deep input.²⁵ When a neuron receives both cutaneous and deep input (eg, skin plus muscle or fascia), it is considered having convergent input. The approximate size and location of the RFs were recorded on a standard outline of the rat body.²⁵ A neuron that responded to the electrical search stimulus but could not be excited by the mechanical stimuli was considered as a neuron without an RF.

2.7. Resting activity

The neuronal resting (ongoing) activity was measured for 1 minute before testing with mechanical stimuli. Neurons showing ≥ 1 impulse/min were considered to be active.

2.8. Data analysis

The PPT and resting activity data are shown as individual values with their respective median; for the recording depth, latency (**Table 1**), and discharge frequency, data are shown as an interquartile range: median (quartile 3–quartile 1) to show a measure of statistical dispersion. The Fisher exact probability test was used to compare the proportion of neurons and the Mann–Whitney *U* (Wilcoxon) test to compare the values of PPT and resting activity. A probability level of less than 5% ($P < 0.05$, 2-tailed; statistical software: GraphPad Prism) was regarded as significant. The effect sizes were determined using Cohen *d* (difference in means divided by pooled SD). An effect size >0.2 was considered as “small,” >0.5 as “medium,” and >0.8 as “large.”⁴

3. Results

3.1. Pressure pain threshold of the low back

Two days after the stress paradigm (**Fig. 1**), the PPT of the MF muscle was determined before the intramuscular NGF injection. There was no significant difference in PPT, suggesting that the stress paradigm induced a latent rather than manifest sensitization. However, animals of the stress group showed a tendency to a lowered PPT ($P = 0.415$; Cohen $d = 0.83$, large effect size; **Fig. 2A**), which was not observed in previously published data (**Fig. 2B**).²⁵

3.2. Responsiveness of dorsal horn neurons to afferent input

Totally, 60 neurons responding to the electrical search stimulus at A-fiber strength were recorded. The recording depth in the dorsal horn ranged from 160 to 960 μm . Their interquartile range was 650 (745–460) μm in control + NGF and 510 (720–380) μm in stress + NGF; no significant differences were found between both groups. Most of the neurons (47 of 60; 78%) were recorded at depths between 400 and 800 μm corresponding to deep dorsal horn laminae IV, V, and VI (refer **Table 1**, recording depth).

The latencies of the electrically evoked action potentials (APs) ranged from 0.8 to 3.4 milliseconds. Their interquartile range was 1.8 (2.1–1.6) milliseconds in control + NGF and 1.8 (2.1–1.8)

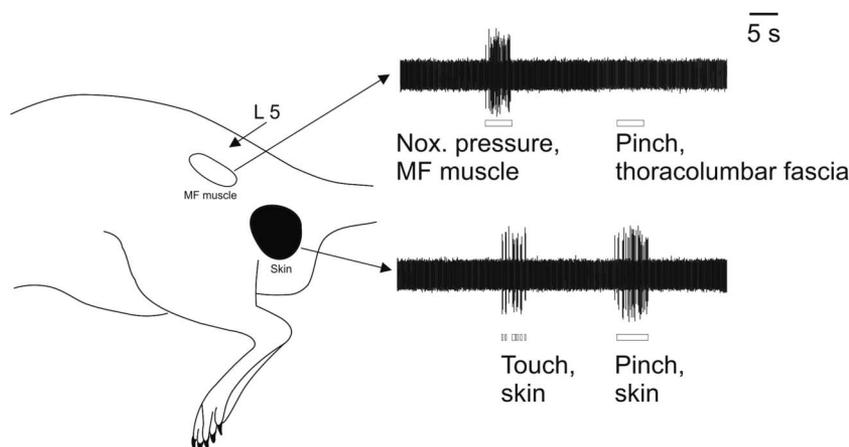


Figure 3. Responses of a single dorsal horn neuron to mechanical stimulation. Example of a convergent neuron having deep input from the lumbar multifidus muscle and input from the skin close to the tail base (stress + NGF group). The scheme shows the location of the receptive fields (RFs) from which the neuron could be activated. Open outline: RF in the multifidus muscles; black area: RF in the skin. (Top) The neuron responded to noxious pressure applied to the muscle but not to pinching the overlying thoracolumbar fascia, indicating an RF in the muscle under the fascia. (Bottom) The neuron responded to touching and pinching the skin. Open bars indicate the time and duration of stimulation. L5: spinous process L5. NGF, nerve growth factor.

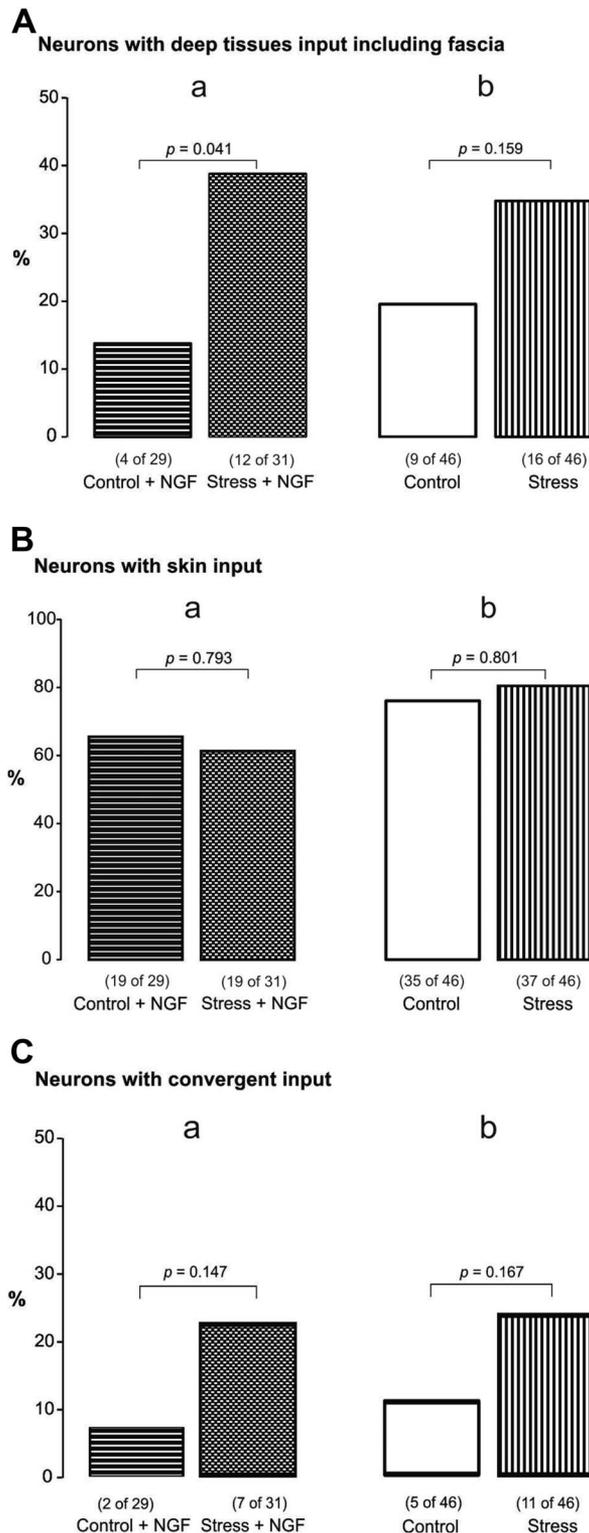


Figure 4. Proportion of neurons having deep, skin, or convergent input. (A) Neurons with input from deep tissues (including fascia) inside and outside the low back (refer Fig. 5). (B) Neurons having input only from the skin. (C) Neurons having convergent input from deep tissues and the skin. (Aa, Ba, Ca) Data from this study (stress + NGF: restraint stress followed by a single NGF injection; control + NGF: handled but not stressed followed by NGF injection). (Ab, Bb, Cb) Recently published data²⁵; stress: restrained animals without NGF injection; control: without restraint and NGF injection. *P* values: Fisher exact test. NGF, nerve growth factor.

milliseconds in stress + NGF. No significant difference was found between the treatment groups. The distance between recording and stimulation electrodes (~35 mm) showed that all neurons tested reacted to A-fiber stimulation. All neurons had myelinated afferent inputs, and the estimated conduction velocities were consistent with ranges in both A-fiber nociceptors and non-nociceptors.¹⁰ Additional C-fiber input was likely activated by the mechanical stimuli used,¹⁸ but this was not verified by electrical search stimuli because we were concerned that such stimuli may induce long-term potentiation.⁶³

Of the 60 neurons recorded, 45 (75%) responded to at least 1 of the mechanical test stimuli used (control + NGF: *n* = 21, stress + NGF: *n* = 24); 15 neurons (25%) responded to the electrical search stimulus but could not be activated by the test stimuli applied to the low back and hind limb (Table 1, without RFs). Of the 45 responding neurons, 36% (16 of 45) received input from deep tissues, 84% (38 of 45) input from the skin, and 20% (9 of 45) had convergent input. Most of the neurons having input from deep somatic tissues (13 of 16; 81%) were located in deep laminae. An example of a neuron having convergent input from the MF muscle and the skin is shown in Figure 3. No significant differences were observed in recording depths between the groups for all types of input (Table 1).

Animals of the NGF group preceded by stress showed a significant increase in the proportion of neurons having input from deep tissues compared with NGF alone (control + NGF: 4 of 29, 14%; stress+NGF: 12 of 31, 39%; *P* = 0.041; Fig. 4A.a). The skin input of all tested neurons did not differ between both groups (control + NGF: 19 of 29, 65%; stress + NGF: 19 of 31, 61%; *P* = 0.793 Fig. 4B.a). The proportion in convergent neurons also increased, but the difference was not significant (control + NGF: 2 of 29, 7%; stress + NGF: 7 of 31, 23%; *P* = 0.147) (Fig. 4C.a). In the previous study,²⁵ stress alone caused a nonsignificant trend in the proportion of neurons having deep input (Fig. 4A.b), skin input unchanged (Fig. 4B.b) and a nonsignificant trend in convergent input (Fig. 4C.b).

An important difference between stressed and unstressed animals was the appearance of new RFs in deep tissues outside the low back, and they mainly appeared in the hip and the entire hind limb (gray areas in Fig. 5A). The number of neurons with RFs in the low back close to the NGF injection site (open outlines in Fig. 5A) increased about 2-fold (control + NGF: 2 of 29, 7%; stress + NGF: 4 of 31, 13%; *P* = 0.671; Fig. 5B.a), whereas the proportion of neurons with RFs located outside the low back increased about 4-fold (control + NGF: 2 of 29, 7%; stress + NGF: 8 of 31, 26%; *P* = 0.081) (Fig. 5C.a). In the previous study with stress alone, no change was seen with RFs inside the low back (Fig. 5B.b), whereas only a 2-fold increase in RFs outside the low back was observed (Fig. 5C.b).²⁵ These data indicate that NGF-induced mild nociceptive input caused a manifest sensitization of DHNs when preceded by stress and such manifest sensitization did not occur after the nociceptive NGF input or stress alone.

3.3. Resting activity of the neurons

Compared with NGF alone, animals of the NGF group preceded by stress showed a significant increase in the proportion of neurons with resting activity (control + NGF: 8 of 29, 28%; stress + NGF: 17 of 31, 55%; *P* = 0.039; Fig. 6A.a). In our previous study, we saw a similar effect of stress alone (stress: 17 of 46,

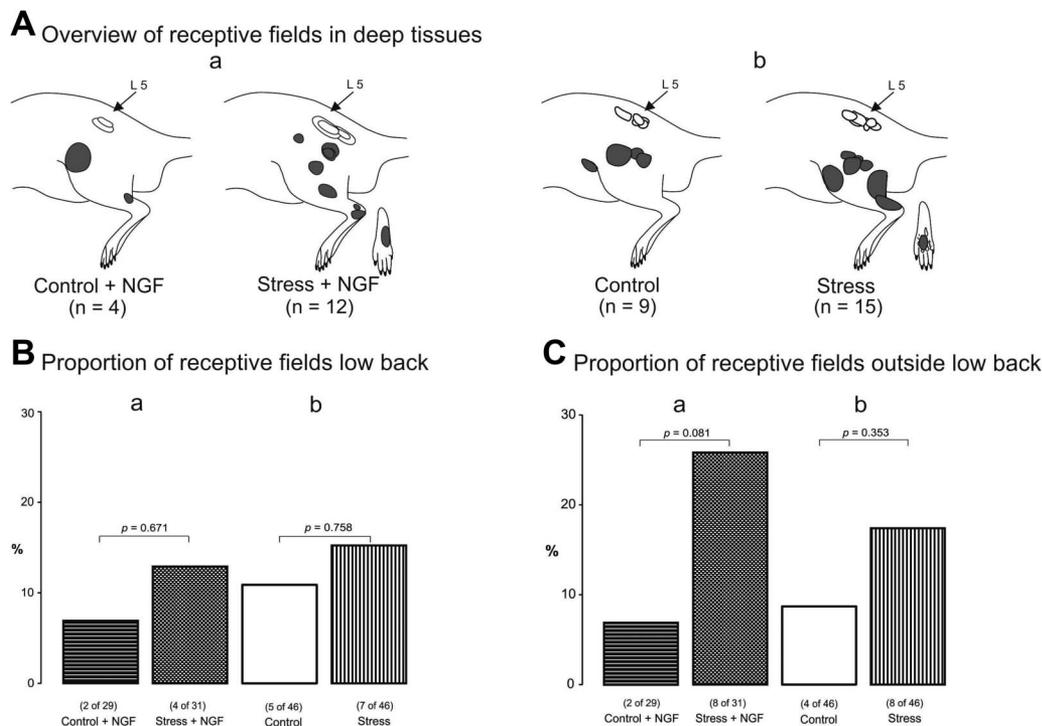


Figure 5. Location of the receptive fields (RFs) in deep tissues. (A) Open outlines show the RFs located in deep tissues of the low back (eg, muscle or thoracolumbar fascia) close to vertebral level L5. RFs in deep tissues outside the low back are marked in gray. (B) The proportion of RFs inside and (C) outside low back. (Aa, Ba, Ca) Data from this study (stress + NGF: restraint stress followed by a single NGF injection; control + NGF: handled but not stressed followed by NGF injection). (Ab, Bb, Cb) Data from a recently published study²⁵; stress: restrained animals without NGF injection; control: without restraint and NGF injection. *P* values: Fisher exact test. NGF, nerve growth factor.

37%; control: 5 of 46, 11%; $P = 0.006$; **Fig. 6A.b**).²⁵ Analysis of resting activity stratified by location of RFs showed us that the manifest sensitization of stress, when combined with a mild nociceptive input, was pronounced on neurons with deep tissue input (control + NGF: 0 of 4 of 29, 0%; stress + NGF: 8 of 12 of 31, 26%; $P = 0.004$; **Fig. 6B.a** and **Table 1**), a similar increase was not seen in neurons with skin input (refer **Table 1**). Only a trend towards increased resting activity in neurons with deep tissue input was observed in our previous study with stress alone (**Fig. 6B.b**). The data from **Figure 6A, B** suggest an additive effect of stress followed by NGF on resting activity of neurons with deep input.

An example of a neuron having resting activity from the stress + NGF group is shown in **Figure 6C**. The mean discharge frequency was highly variable, and the firing pattern was irregular both within and across neurons. Hence, we did not see a significant difference between NGF alone and stress + NGF, neither when including all—active and silent—neurons and their interquartile ranges (control + NGF: 0 [1–0]; stress + NGF: 1 [9–0] impulse/min; $P = 0.065$) nor active neurons alone (control + NGF: 53 [114–2]; stress + NGF: 8 [44–3]; $P = 0.559$; **Fig. 6D**).

4. Discussion

Repeated restraint stress followed by a single NGF injection into the MF muscle significantly increased the percentages of DHNs having resting activity and input from deep soft tissues, especially outside the low back. Thus, both hypotheses were confirmed. An increased deep tissue input was not present after a single injection of NGF,²² and only a nonsignificant trend towards an increase was found after repeated restraint stress alone.²⁵ These data suggest that restraint stress or a single NGF injection

induced a state of latent sensitization, whereas the combination of both induced a manifest sensitization.

4.1. Latent vs manifest sensitization of dorsal horn neurons by stress or nociceptive input

Previous studies have shown that the responsiveness of DHNs to muscle input increased after 2 NGF injections at a 5-day interval, but not after a single NGF injection.^{22,24,62} These findings led to the concept of latent sensitization caused by the first NGF injection that can be uncovered by the second one. In this study, the responsiveness of DHNs to deep input increased significantly in stressed animals after a subsequent mild nociceptive input (NGF injection), whereas stress alone had previously induced only nonsignificant changes in DHN responsiveness.²⁵ This finding suggests that restraint stress caused a state of latent sensitization or priming and these primed neurons had an enhanced susceptibility to developing a manifest sensitization in response to a subsequent NGF injection.

The manifest sensitization was input specific in that the latent sensitization was challenged with mild nociceptive input from deep tissue, and only DHNs with deep tissue input were upregulated. Upregulation included convergent neurons, but the overall cutaneous input was unchanged in this and other studies.^{20,22,25,62} Cutaneous afferents have highly effective synaptic connections on DHNs and might have a ceiling effect for additional sensitization.²⁵

The appearance of new RFs outside the low back indicates that DHN responsiveness was enhanced also for deep tissue input that had not been activated by the NGF injection. This finding supports the conclusion that manifest sensitization occurred at DHNs and not in the periphery. This pattern is

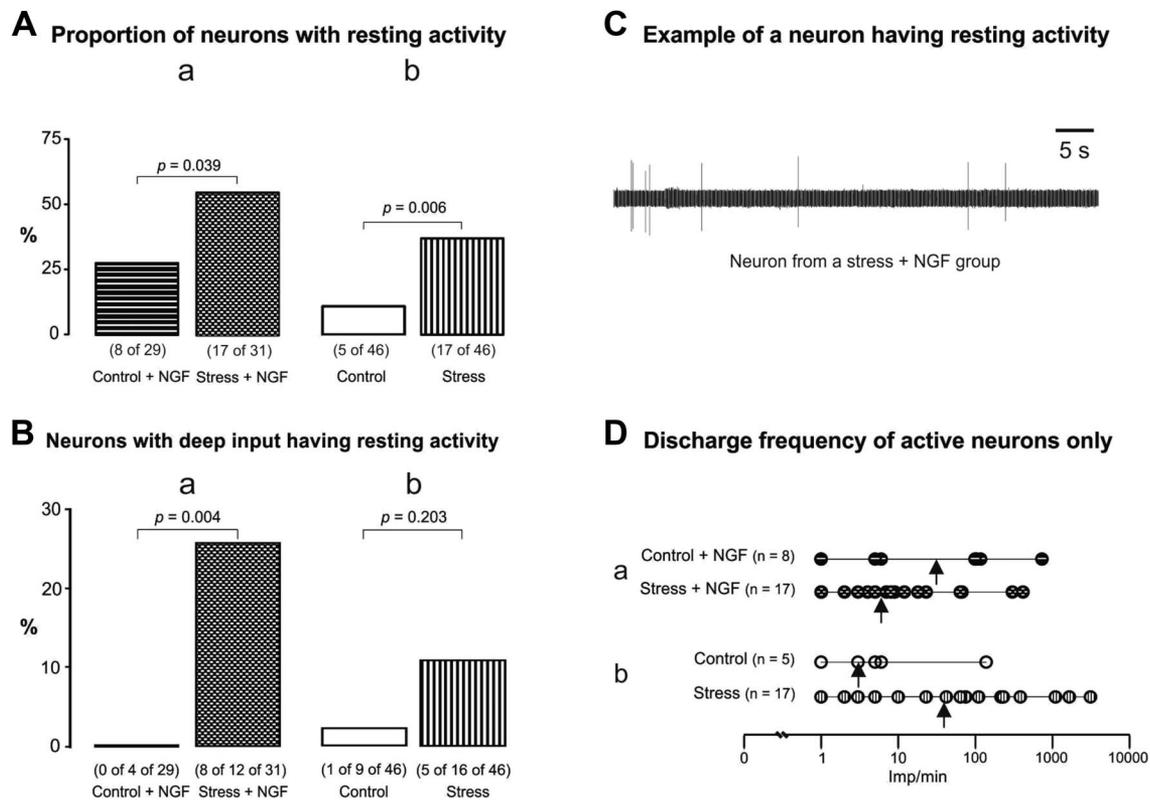


Figure 6. Resting activity of dorsal horn neurons. (A) The proportion of neurons having a discharge activity ≥ 1 impulse/min. (B) Neurons with deep tissue input and having resting activity. (C) Original registration from a neuron having resting activity. (D) Discharge frequency of active neurons only. Arrowheads indicate the median discharge frequency of the active neurons. (Aa, Ba, C) Data from this study (stress + NGF: restraint stress followed by a single NGF injection; control + NGF: handled but not stressed followed by NGF injection). (Ab) Recently published data,²⁵ (Bb) reanalyses of published data²⁵ based on RFs location and resting activity; stress: restraint stress animals without NGF injection; control: without restraint stress and NGF injection.; *P* values in A and B: Fisher exact test. NGF, nerve growth factor.

reminiscent of pain referral from deep tissues, which is referred to other deep tissues and not to the skin,^{12,19,21} similar to the “pseudoradicular” pain in the proximal leg of patients with LBP.¹³

4.2. Possible mechanisms and pathways underlying stress and nerve growth factor-induced changes

New RFs are an important sign of central sensitization; they may emerge based on increased synaptic strength of ineffective synapses on DHNs that normally only induce sub-threshold postsynaptic potentials but no APs.^{11,27,29,56} It is known that DHNs have a subliminal fringe around their RFs and central sensitization can recruit this fringe into the RFs from which the neurons respond with APs.^{41,58} There are also so-called “silent” synapses in the central nervous system, which express NMDA receptors only and, hence, respond to glutamate released only after preceding depolarization by another input; unsilencing of such synapses to lamina I neurons has been found for an inflammatory pain model.⁵³ We recently demonstrated by intracellular recordings in a model of muscle pain that hypersensitivity of the DHNs to peripheral input was due to both unmasking of silent synapses and increased synaptic strength.¹⁸

In a rat model of temporomandibular pain, stress has been found to sensitize DHNs in deep laminae rather than superficial laminae.³⁹ Primary afferents from muscles project to both superficial and deep DHNs,^{5,35} but input from low back muscles is more prominent in deep laminae.⁵⁰ Although our search included both superficial and deep

DHNs, most neurons were found around lamina V, ie, in a region where low back input may be modulated by stress. Such modulation may be intraspinal through microglia activation or astrocytes, both of which may be activated by stress^{28,32,39} and are relevant for latent and manifest sensitization in our back pain model.⁶² Chronic stress can also enhance the release of proinflammatory cytokines⁵⁵ that might contribute to the observed latent sensitization.

Descending facilitation through a brainstem loop contributes to central sensitization and enhanced excitability of DHNs in neuropathic pain models.^{48,62} These descending pathways can be activated by stress through an excitatory pathway from the medial hypothalamus to rostral ventral medulla oblongata^{17,34} and onward to the dorsal horn; this phenomenon has been called “stress-induced hyperalgesia.” Increased descending facilitation or decreased descending inhibition can both enhance the excitability of DHNs.¹⁷ In nociceptive DHNs in lamina V, the descending inhibition had a stronger effect on input from deep tissues than on the cutaneous input to the same neurons.⁶² This may explain why stress is a predisposing factor for myofascial pain rather than cutaneous pain in humans.

The inhibitory or facilitatory brainstem controls usually affect the entire spinal cord⁴⁷; this predicts a spatial spread of central sensitization when it is mediated through these mechanisms.⁵⁴ The new RFs in deep tissues in this study were mostly located outside the stimulated low back region, which is consistent with the involvement of a brainstem loop. Repeated acid injections into limb muscles also induce central sensitization through such a brainstem loop, mimicking aspects of CWP in humans.⁷ Because our NGF injection model is dependent on microglia signaling,⁶² the spatial spread of sensitization

may also be due to diffusion of glia-derived mediators, as has been shown for heterosynaptic spinal LTP.^{30,46,47,60} Microglia can be activated by primary afferent input through the CX3CL1 (fractalkine) to CX3CR1 pathway.^{3,40,60} Synergistic action of stress and nociceptive input on glia has also been observed.⁴² Both spinal and supraspinal mechanisms are likely to be involved in this synergistic action between stress and a mild nociceptive input by NGF.

4.3. Behavioral signs of nociceptive priming by stress

The PPT measurements showed no significant difference in pressure pain sensitivity between the groups similar to our previous study.²⁵ But, we saw a larger effect size in this study, which was absent in the previous study. This may be due to subtle differences in experimenter and environment: (1) sex of the experimenter (male in this study), (2) different restrainer, and (3) different animal facilities. These behavioral data suggest that our stress paradigm primed the DHNs sufficient enough to induce a manifest spinal sensitization with additional mild nociceptive input.

4.4. Limitations of the study

- (1) Restraint stress included a component of motor inactivity, which is another risk factor in human LBP in addition to stress; a recent study in mice had shown differences between vertical and horizontal immobilization.³¹
- (2) A biomarker for stress was not assessed in this study, but a significant increase in fecal corticosterone metabolites and lowered body weight was observed in our previous study.²⁵
- (3) Owing to the experimental design, no behavioral data after NGF injection were available to test for behavioral correlates of the electrophysiological data showing manifest sensitization.
- (4) We cannot distinguish to what extent mechanical nociceptive input of the injection or receptor-mediated effects of NGF triggered the transition from latent to manifest sensitization; in previous studies, vehicle injections were ineffective.^{22,62}
- (5) Experimenters were not blinded. To minimize investigator bias, an electrical search stimulus was used for unbiased sampling of DHNs and the search for RFs strictly followed a standard protocol (refer section 2.6).

5. Conclusions

These data show that restraint stress induces a latent sensitization of DHNs and primes for manifest sensitization by subsequent NGF injection as a model of mild nociceptive input from low back muscles. The manifest sensitization of DHNs was indicated by increased afferent input from deep soft tissues, an expansion of deep RFs into the hind limb, and increases in resting activity in those neurons. These neurophysiological parameters suggest a synergy between mild noxious events in low back muscles and stress and may reflect the neuronal background of spontaneous and evoked pain in patients with LBP.

Disclosures

The authors have no conflicts of interest to declare.

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