PAIN® 152 (2011) 1856–1863

www.elsevier.com/locate/pain

NGF enhances electrically induced pain, but not axon reflex sweating

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Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

ARTICLE INFO

Article history:
Received 17 November 2010
Received in revised form 23 March 2011
Accepted 1 April 2011

Keywords:
Sweat
QSART
Sudomotor
Nociceptors
Axonal excitability
Activity-dependent slowing

ABSTRACT

High-affinity receptors for nerve growth factor (NGF) are found on nociceptors and sympathetic efferents. NGF is known to sensitize nociceptors, increase innervation density, and fire frequency of sympathetic fibers. We explored axonal sensitization of afferent and efferent fibers following intracutaneous injection of NGF in human and pig skin. In humans, frequency-dependent (5, 20, 100 Hz) electrically induced pain was assessed 1, 3, 7, 21, and 49 days post injection. Sweat output was recorded in parallel using the quantitative sudomotor axon reflex test (QSART). Electrically induced pain ratings (7.5 mA for 30 s) significantly increased at the NGF sites for 5 Hz (numeric rating scale [NRS] 6 ± 0.5 vs 3.7 ± 0.4), 20 Hz (NRS 7.2 ± 0.4 vs 5 ± 0.5), and 100 Hz stimulation (NRS 6.9 ± 0.4 vs 5.4 ± 0.3) at day 21, and also for 5 Hz at day 49 (NRS 5.4 ± 0.4 vs 3.8 ± 0.3). Electrically evoked QSART increased frequency dependent, but was not altered by NGF throughout the entire observation period (average QSART at 5 Hz: 3 mL/h/m^2, 20 Hz: 9 mL/h/m^2, 100 Hz: 10 mL/h/m^2). Similarly, NGF did not change the activity-dependent slowing of conduction of sympathetic efferents (6 ± 2% vs 5.1 ± 1.5%, for 3 minutes, 2 Hz) in pig single-fiber recordings. In parallel to the increased pain ratings recorded in humans, activity-dependent slowing of mechano-insensitive nociceptors was reduced by NGF (18.1 ± 2% vs 29 ± 1.4%). In summary, axonal sensitization of nociceptors by NGF could underlie the hyperalgesia to electrical stimulation. Enhanced responses were limited to nociceptors, as no sensitization was found in sympathetic efferent neurons.

1. Introduction

Administration of nerve growth factor (NGF) to human skin has been shown to cause acute sensitization to thermal stimuli but sustained hyperalgesia to mechanical stimuli [39]. Acute heat sensitization most likely involves transient receptor potential vanilloid 1 receptor translocation and phosphorylation [53]. The mechanisms by which NGF evokes mechanical hyperexcitability with a peak at 3 weeks and lasting for several weeks are not fully understood, but are linked to the expression of high-affinity (trkA) [13,22,31] and low-affinity (p75) NGF receptors [38,52] on primary sensory neurons. Long-lasting sensitization processes may involve NGF/trkA-receptor internalization, transport of the complex to the dorsal root ganglion with consecutive protein expression changes, and anterograde protein transport to the sensory ending [21,51]. NGF can directly modulate expression and activity of transducer molecules like transient receptor potential cation channel, member A1 [10] or acid-sensing ion channel 3 [30], or other members of the degenerin/epithelial N+ channel family [11]. Beyond modulating transduction, NGF may also induce an alteration of axonal protein expression, for instance, sodium channels [3,14,18,36]; and modulate action potential generation and conduction, thereby possibly leading to sensitivity changes and hyperalgesia. Altered kinetics of sodium channels [43] may also participate in prolonged NGF-evoked sensitization processes.

TrkA receptors are present on sympathetic efferent neurons [48], and NGF modulates voltage-gated sodium and potassium channels on sympathetic efferents, thus changing their repetitive firing rate via trkA- and p75-signaling pathways [27,28]. The basic aim of this study was to investigate NGF-induced axonal sensitization in nociceptive afferent and sympathetic efferent unmyelinated nerve fibers. Electrical stimulation delivered transdermally was used to excite the axons eluding activation of sensitized sensory endings. Subjects' pain perception upon high-frequency electrical stimulation was recorded psychophysically in NGF and NaCl pretreated skin. Sympathetic function was assessed in parallel by the electrically induced quantitative sudomotor axon reflex test (QSART) [26,42]. As a translational approach, single-fiber recordings from nociceptors and sympathetic efferents were performed in pig skin in vivo, and NGF-evoked effects on activity-dependent

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0304-3959/$36.00
doi:10.1016/j.pain.2011.04.002

slowing (ADS) of conduction were assessed at 3 weeks after injection, when mechanical and electrical hyperalgesia was maximal in humans [39].

2. Methods

The local Ethics Committee of the Medical Faculty Mannheim, University of Heidelberg, approved the experimental procedure on human volunteers according to the Declaration of Helsinki. The animal protection authorities of Baden-Württemberg, Germany, and the central animal research unit at the University of Heidelberg approved the study protocol and experimental procedure performed in pigs.

Sixteen healthy volunteers (8 male, 8 female, average age 36 ± 9 years) were recruited, informed about the purpose and time course of the study, and requested to sign an informed consent form. All subjects were familiarized with the methods used to activate sympathetic efferent neurons and to quantify the corresponding sudomotor responses.

2.1. NGF administration and experimental protocol in humans

Intradermal injections of 1 μg human recombinant lyophilized NGF (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) dissolved in 50 μL saline, were performed into the central volar forearm using insulin syringes (30 G × 8 mm; Becton Dickinson, Franklin Lakes, NJ, USA). As control, 50 μL NaCl 0.9% (Braun, Melsungen, Germany) was injected into the contralateral forearm. Injection sites were labeled with a felt-tip pen and volunteers were investigated at day 1, 3, 7, 21, and 49 after the NGF administration. Electrical stimuli (150 pulses at 7.5 mA, 0.5-ms pulse width) were delivered transdermally for 30 s by a pair of self-adhesive 3 × 10-mm electrodes (Pierenkemper, Wetzlar, Germany) attached to the skin at the injection sites, and connected to a constant current stimulator (DS7A, Digitimer Ltd, Hertfordshire, UK) triggered by a pulse generator (Pulsgenerator PG1; Rimkus, Parsdorf, Germany). Prior to stimulation onset, perception thresholds for electrical stimuli were determined at both the NGF and NaCl sites (pulses of 1 Hz, 0.5-ms pulse width). Thereafter, for stimulation, 150 electrical pulses were delivered in randomized order at frequencies of 5, 20, and 100 Hz. For investigating the frequency of 5 Hz, the 150 pulses were administered continuously throughout the 30-s stimulation period. For investigating higher frequencies (20 and 100 Hz), bursts of 10 pulses were delivered every 2 s for 30 s. Thereby, the frequency of the 10 pulses within each burst was set at 20 or 100 Hz, respectively. The volunteers were instructed to estimate maximum pain perceived within the stimulation period on an 11-point numeric rating scale (NRS) with the endpoints 0 (no pain) and 10 (worst pain imaginable).

Electrically evoked axon reflex-mediated sweat response was recorded as described previously in detail [34,42]. In principle, a circular capsule (diameter 2.5 cm) was placed above the stimulation electrodes and continuously perfused with dry air at 6 L/h. The electrically induced sweat evaporation increased the humidity of the perfusing air. This increase was measured by a humidity sensor (HygroClip-SC04; Rotronic GmbH, Ettlingen, Germany) connected to a control unit (HygroLab 2, Rotronic GmbH) after the skin passage. Sweat output was calculated offline in mL/h/m^2 according to the recorded humidity, the flow rate, and the skin surface area covered by the capsule.

Furthermore, systemic sympathetic responses were investigated by the sweat output recorded from nonstimulated capsules placed on the forearm 20 cm distal to the stimulated site, well outside the axon reflex area.

Pain ratings and sweat output were assessed in 2 consecutive sessions at either the NGF- or the NaCl-pretreated forearm. Responses to each pulse frequency were assessed in triplicate with time intervals of 8 minutes between each session.

2.2. NGF administration and experimental protocol in pigs

In vivo electrophysiological recordings were performed after NGF treatment in 7 male domestic pigs (*Sus scrofa*, German landrace; age 2–4 months; median bodyweight 25 kg). Animals were housed for 1 week before the start of the experimental procedures, fed twice daily, and had access to water ad libitum. Food was withdrawn on the day preceding the surgical interventions.

NGF (Sigma, Deisenhofen, Germany) (10 μg) was dissolved in 3 mL saline and the entire volume was injected in doses of 20 μL (30-G insulin syringes; BD, Heidelberg, Germany) to one hind limb of the sedated (2 mg/kg azaperone and 1 mg/kg midazolam) pig. NGF was administered to the medial aspect of the hind limb, covering an area of 10 × 15 cm between the knee (proximal) and the ankle (distal). A distance of 1 cm between the injections was kept constant. Three weeks after NGF administration, nerve fiber recordings were performed as described previously [35]. In brief, pig anesthesia was induced with 2 mg/kg propofol and maintained by continuous pentobarbital infusion (8–14 mg/kg/h). Muscle relaxation was maintained by continuous succinylcholine infusion (8 mg/kg/h) preceding a 0.5-mg/kg rocuronium intravenous injection. The saphenous nerve was exposed at mid-thigh on a length of about 6 cm, and action potentials were recorded using the teased fiber technique [29]. Nerve signals were amplified (Model 5113; Ametek Inc, Oak Ridge, TN, USA), filtered, audio-monitored, and displayed on an oscilloscope. Individual C units were identified according to their time-locked discharge upon electrical pulses (20 mA, 0.5-ms pulse width, DS7A, Digitimer Ltd) delivered to the receptive field by means of 2 noninsulated microneurography electrodes (FHC Inc, Bowdoin, ME, USA). Current intensity was adjusted to 1.5 times the electrical thresholds determined during continuous electrical stimulation at 0.25 Hz. Mechanical and thermal responses were tested for C-nociceptor classification. The criteria for classification into mechano-sensitive (CM) and mechano-insensitive (CMi) C nociceptors have been described previously [35]. In brief, low-threshold C-touch fibers were screened by their positive response to non-noxious touch stimuli. Fibers being brush-negative but responsive to 150-mN stimulation were classified as mechano-sensitive nociceptive units (CM). Afferent C fibers nonresponsive to 150-mN stimulation were classified as CMi. Sympathetic efferent units (SYMP) were identified by their unresponsiveness to sensory (mechanical or thermal) stimuli and by the typical reversal in their activity-dependent conduction velocity slowing at 2-Hz stimulation [5], as described below. Following a resting period of 2 minutes, units were stimulated for 3 minutes at 2 Hz, and total latency changes (ADS) were recorded [41]. Activity-dependent response latencies were normalized to the initial latency recorded upon the first stimulus after the 2-minute resting period and depicted as % increase. If conduction failure occurred during the 2-Hz stimulation period, the current intensity was increased to ensure that nerve fibers maintained firing. Those fibers that did not follow the 2 Hz for 3 minutes, even at a maximally administered cutoff current intensity of 60 mA, were omitted from the analysis of total ADS. However, all fibers could follow the first 10 pulses of the 2-Hz stimulation and therefore, ADS of these 10 pulses (ADS_{10}) was analyzed separately for units with and without conduction failure. Control data serving for the comparison to NGF were obtained from nerve-fiber recordings of 24 naïve animals. The generated action potentials were displayed, processed, recorded, and analyzed offline using dedicated software (DAPSYS 7.0, [47]).

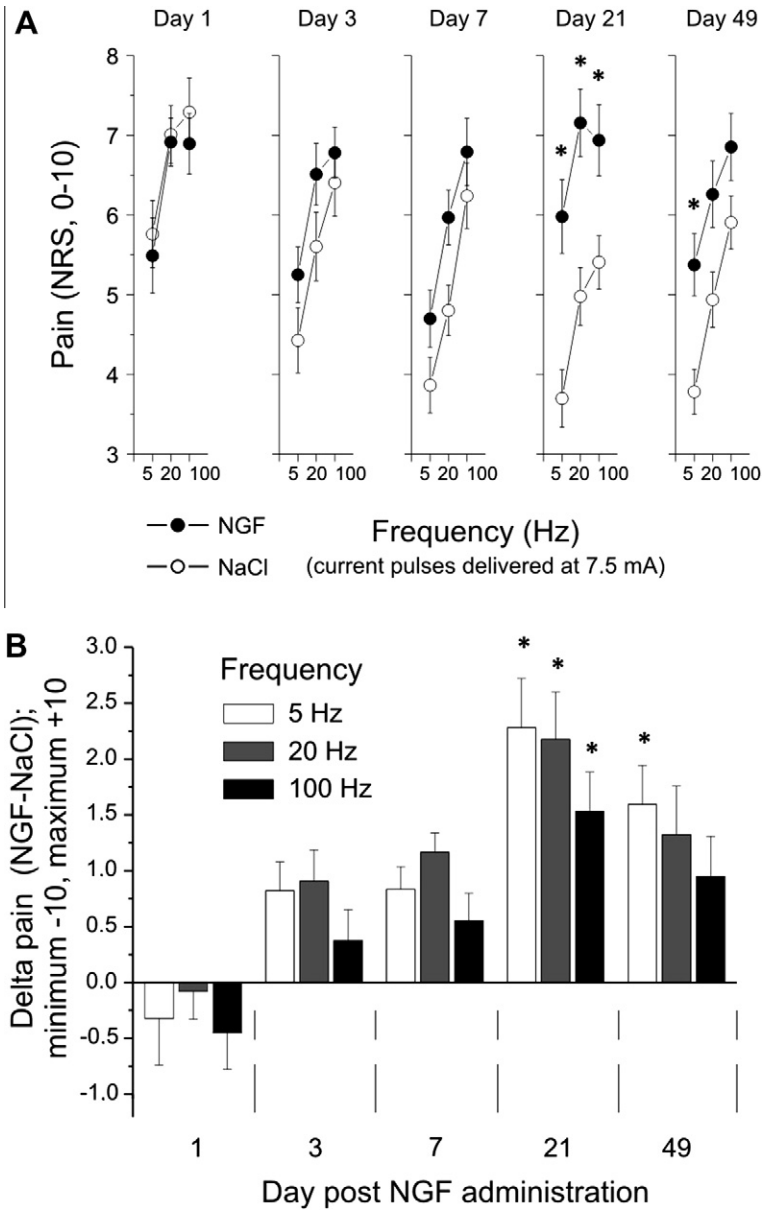

Fig. 1. Electrically induced pain (numeric rating scale, NRS) recorded at the nerve growth factor (NGF) and NaCl sites (A) and depicted as difference (delta-Δ-pain) between NGF- and NaCl-pretreated skin (B). Current pulses (7.5 mA) were delivered transdermally at frequencies of 5, 20, and 100 Hz at days 1, 3, 7, 21, and 49 after NGF administration. Positive values of delta-Δ-pain indicate enhanced responses at the NGF site. In comparison to NaCl, significantly increased pain was recorded at day 21 for all frequencies ($P < 0.0001$, Scheffé post hoc) and at day 49 for 5 Hz ($P < 0.005$, Scheffé, marked by asterisks).

2.3. Statistics

Data were compiled in Microsoft Office Excel 2003 (Microsoft Corporation, Redmond, WA, USA) and analyzed using Statistica 7.0 software package (Statsoft, Tulsa, OK, USA). Pain and sweat output upon electrical stimuli were analyzed by repeated-measures analysis of variance (ANOVA). Between-subjects factors were "NGF" vs "NaCl" and within-subjects factors were "day of investigation" and "stimulus frequency." Scheffé post hoc repeated measures with $P < 0.05$ identified significant differences within the factors.

Data of single-nerve fiber recordings (spike discrimination and latency measurements) were processed offline (DAPSYS 7.0) and exported to Excel. If the stimulation current had to be increased, the "relative stimulation intensity" was determined by calculating the ratio between the current intensity required to maintain conduction and the electrical threshold. Latencies recorded at 2-Hz stimulation were normalized as percentage of the first latency recorded after the 2-minute pause. Relative stimulation intensities and latency changes were analyzed by multi-ways ANOVA and least significant difference (LSD) post hoc test using "treatment" (NGF vs control), "unit type" (CMi, CM, SYMP), and "status of conduction block" as categorical factors.

Pain ratings (Fig. 1) are depicted as (A) raw values and (B) differences (Δ-) calculated between the NGF and NaCl sites (means ± SEM). Sweat output values (Fig. 2) are depicted as increase of sweat volume (mL) above baseline evaporated per hour and m^2 skin surface (mean ± 95% confidence interval). Nerve fiber latency changes recorded from the pig saphenous nerve (Fig. 3) are given as normalized increase from the initial latency (%) and depicted as means ± SEM.

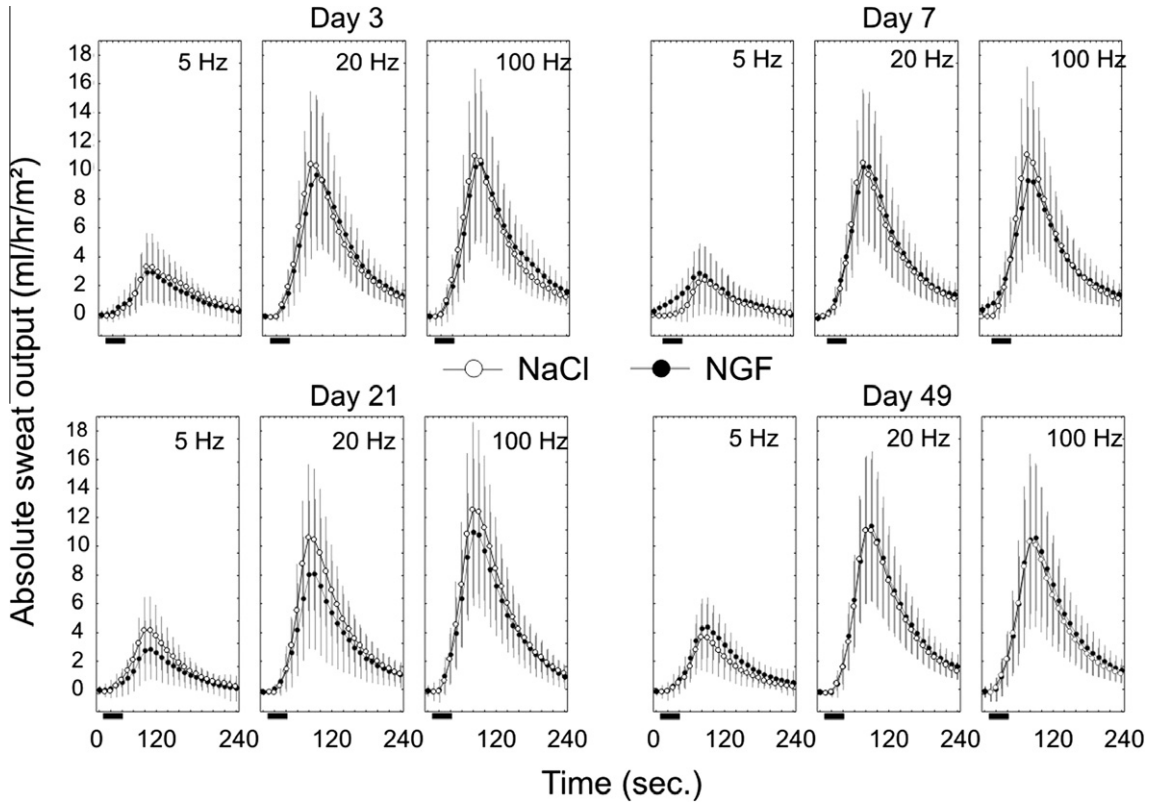

Fig. 2. Quantitative sweat output (mL/h/m^2) recorded at the NaCl (open circles) and nerve growth factor (NGF) (solid circles) pretreated forearm skin in response to 150 current pulses (7.5 mA) delivered within 30 s (black bar) at 5, 20, and 100 Hz, respectively. No significant impact on sudomotors was identified between the NGF and NaCl sites throughout the observation period of 49 days.

3. Results

3.1. Electrically induced pain after NGF

Perception thresholds to electrical stimuli were on average (days 1–49) 0.91 ± 0.06 mA at the NGF and 0.89 ± 0.05 mA at the NaCl sites (means ± SD, n.s., ANOVA, data not shown). Pain ratings increased significantly by NGF treatment ($P < 0.0001$, main effect "NGF," ANOVA) and over time of investigation ($P < 0.003$, main effect "day," ANOVA), and in a stimulus frequency-dependent manner ($P < 0.0001$, main effect "Hz," ANOVA) (Fig. 1). In comparison to 5 Hz, high-frequency stimuli delivered at 20 Hz were more painful in NGF-treated ($P < 0.03$, Scheffe) but not in NaCl-control skin ($P > 0.1$, Scheffe). Stimuli of 100 Hz were more painful in both NaCl and NGF skin at each time point of investigation ($P < 0.01$, Scheffe post hoc) (Fig. 1A). Throughout the 49-day observation period, electrically induced pain ratings at the NaCl injection sites were: NRS 4.3 ± 1.6 at 5 Hz, NRS 5.5 ± 1.6 at 20 Hz, and NRS 6.3 ± 1.6 at 100 Hz (means ± SD). In contrast, at the NGF-treated skin sites, pain was NRS 5.8 ± 1.8 at 5 Hz, NRS 6.4 ± 1.6 at 20 Hz, and NRS 6.6 ± 1.6 at 100 Hz. Differences of perceived pain (Δ pain) between the NGF- and NaCl-treated sites were calculated for each administered frequency and day of investigation (Fig. 1B). Significantly enhanced Δ pain was recorded at day 21 for all investigated frequencies (on average, $P < 0.0001$, Scheffé post hoc). Furthermore, at day 49, significantly increased pain was recorded at 5 Hz ($P < 0.001$, Scheffé post hoc) after NGF treatment.

3.2. Sudomotor responses after NGF

Electrical stimulation of sympathetic efferent neurons evoked an increased sweat output in a frequency-dependent manner ($P < 0.0001$, ANOVA). Peak maximum responses were recorded at approximately 60 s after the end of stimulation, which were throughout the observation period (days 1–49), on average, 3.7 ± 2.2 mL/h/m^2 (NaCl) and 3.0 ± 2.5 mL/h/m^2 (NGF) for 5 Hz, 10.5 ± 4.8 mL/h/m^2 (NaCl) and 9.3 ± 4.7 mL/h/m^2 (NGF) for 20 Hz, and 11.5 ± 5.1 mL/h/m^2 (NaCl) and 9.6 ± 4.7 mL/h/m^2 (NGF) for 100 Hz stimulation (Fig. 2). No significant differences were identified between NGF- and NaCl-treated sites ($P > 0.9$, ANOVA) or between the days of investigation ($P > 0.8$, ANOVA).

3.3. Differential NGF effects on the axonal characteristics of nociceptive and sympathetic porcine C-fibers

In the pig skin, electrical thresholds of mechano-insensitive nociceptors are higher (~12 mA) than for sympathetic efferents (~7 mA) [35]. The present results indicate that some porcine nociceptors fail to conduct when stimulated at higher rates. In Fig. 3A (left panel), the activity-dependent increase in response latency ("slowing," ADS) of a mechano-insensitive nociceptor is depicted. The unit failed to follow the 2-Hz input for 3 minutes even though the stimulation intensity had been increased to 12-fold its electrical threshold. In contrast, one sympathetic fiber (right panel) with characteristically low ADS (~7%) did not fail conduction when stimulated at only 1.5-fold of its electrical threshold. Notably, the stimulation intensity required to ensure regular spike responses throughout the 2-Hz stimulation period correlates to the amount of ADS of conduction velocity of all C fibers recorded from untreated skin ($r = 0.534$; $P < 0.00001$; n = 62; Spearman's correlation; Fig. 3B). As shown before [35], CMis display the highest rate of ADS upon 2-Hz stimulation. At 3 weeks after NGF administration, the ADS of CMi units (18.1 ± 2%; n = 9) was significantly lower as compared to CMi fibers in control skin (29 ± 1.4%; n = 17;

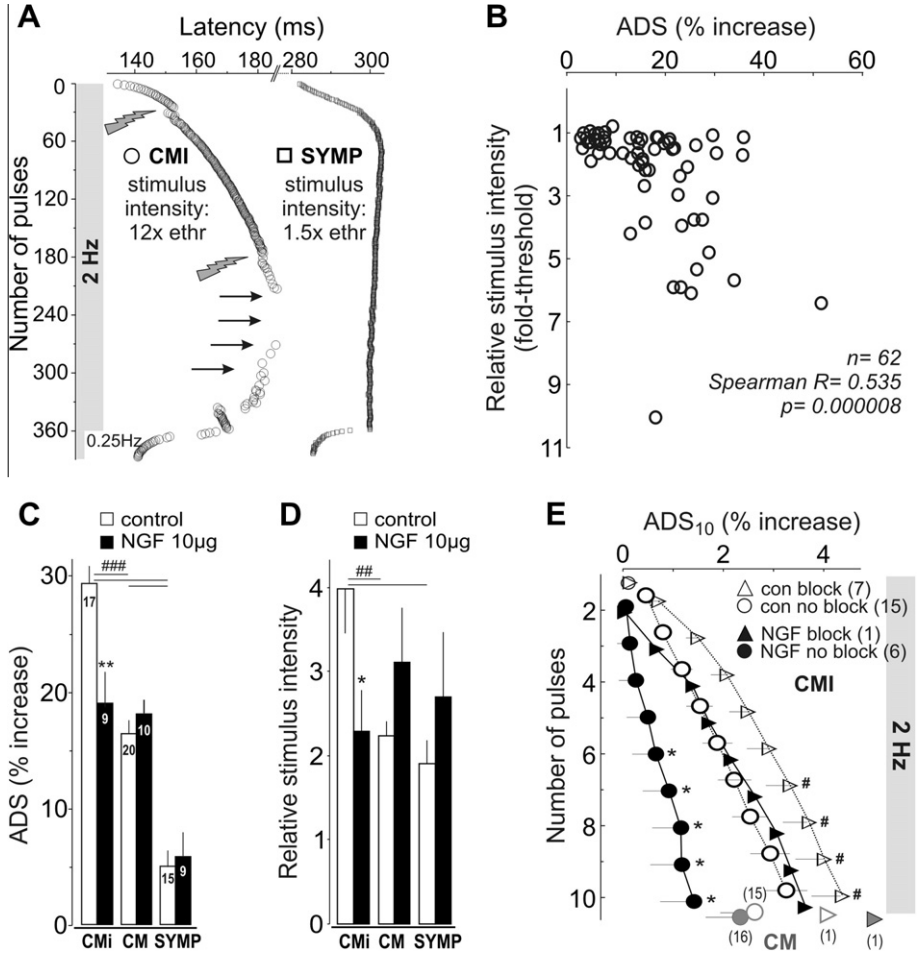

Fig. 3. (A) Specimen of a mechano-insensitive nociceptor (CMi) and a sympathetic efferent unit (SYMP) recorded during electrical stimulation at 2 Hz for 3 minutes. The numbers of delivered pulses are depicted from top to bottom (ordinate) and the corresponding activity-dependent response latencies (ms) from left to right (abscissa on the top). Note that temporary conduction failures (indicated by the "current arrow") were partially compensated by increasing the stimulation intensity up to the 60-mA cutoff (equivalent to 12-fold threshold of this fiber specimen). Nevertheless, after about 2 minutes of stimulation, conduction failures (arrows) persisted despite maximal current intensity. The unit resumed conduction during the recovery at 0.25-Hz stimulation. In contrast, the SYMP unit stimulated at 1.5-fold of its electrical threshold did not block conduction during the 2-Hz stimulation. (B) Activity-dependent slowing (ADS; given as total percentage increase of the response latency) correlates with the relative stimulation intensity (ordinate, given as fold threshold) required to avoid conduction failure during the 2-Hz stimulation. (C) ADS of CMi, mechano-sensitive C-nociceptors (CM), and sympathetic efferent fibers (SYMP) recorded from untreated (control, white columns) and nerve growth factor (NGF; black columns) pretreated pig saphenous nerve. Values are given as relative increase of response latency at the end (third minute) of the 2-Hz stimulation. In the untreated control skin, CMi-nociceptors reveal significantly larger ADS when compared to CM and SYMP units ($P < 0.001$, LSD post hoc test, marked by hashes). At 3 weeks after 10 µg intradermal NGF administration, ADS of the CMi-nociceptors was changed ($P < 0.01$, LSD, marked by asterisks). The number of recorded units is displayed in each column. (D) Relative stimulus intensity (fold threshold) required to avoid conduction failure in CMi, CM, and SYMP units during continuous electrical stimulation (2 Hz, 3 minutes). In untreated control skin (white columns), significantly higher intensities were needed for CMi-nociceptors in comparison to CM and SYMP units ($P < 0.01$, LSD post hoc test, marked by hashes). At 3 weeks after 10 µg NGF (black columns), the stimulus intensity was reduced significantly in CMi units ($P < 0.05$, marked by asterisks). (E) Activity-dependent slowing during 10 pulses delivered at 2 Hz (ADS_{10}) recorded from CMi-nociceptors in untreated ("con," open symbols) and NGF pretreated skin (solid symbols). CMi units that fail conduction during the 3-minutes stimulation at 2 Hz (maximum 60 mA) are depicted in triangles ("block") and those following the pulses without failure are depicted in circles ("no block"). Nociceptors with conduction block reveal significantly higher ADS as compared to units without conduction failure ($P < 0.001$, marked by hashes). At 3 weeks after 10 µg NGF, all CMi units display significantly less ADS as compared to those recorded from control skin ($P < 0.001$, marked by asterisks). ADS recorded from mechano-sensitive (CM) nociceptors are depicted in gray and shown for the 10th pulse of the 2-Hz stimulation only. The number of recorded CM units is shown in parentheses.

$P < 0.01$, ANOVA), but was identical to the ADS of CM nociceptors (18.5 ± 1.9%; n = 10; $P > 0.9$, ANOVA; Fig. 3C). ADS of sympathetic efferent C fibers (SYMP) was not changed at 3 weeks after NGF treatment (6 ± 2%; n = 9) as compared to untreated control skin (5.1 ± 1.5%; n = 15; $P > 0.9$, ANOVA; Fig. 3C). Similarly, as depicted in Fig. 3D, CMi nociceptors required significantly higher stimulation intensities to ensure regular spike responses at 2 Hz (4 ± 0.5 times their electrical threshold) when compared to CM nociceptors (2.2 ± 0.2 times electrical thresholds; $P < 0.01$; ANOVA) or sympathetic efferent fibers (1.9 ± 0.3 times electrical thresholds; $P < 0.01$; ANOVA, Fig. 3D). At 3 weeks after NGF, relative stimulation intensities decreased significantly in CMi nociceptors (2.3 ± 0.5; $P < 0.05$, ANOVA). Among CM nociceptors and sympathetic units, the stimulus intensity tended to increase after NGF, but differences did not reach statistical significance (Fig. 3D). In 7 of 22 (31.8%) control CMi units, a conduction failure occurred that could not be overcome by augmented stimulation intensities. These 7 CMi units displayed significantly more ADS after 10 pulses at 2-Hz stimulation (ADS_{10}) as compared to the 15 CMi units without conduction block ($P < 0.001$; ANOVA, open symbols in Fig. 3E). Consistent with Fig. 3C, NGF treatment also reduced ADS_{10} in all nonblocked CMi nociceptors (solid circles, n = 6) as compared to the nonblocked CMi units recorded from control skin (open circles, $P < 0.001$, ANOVA, Fig. 3E). Moreover, NGF treatment tended to reduce the proportion of CMi nociceptors with conduction failure (1 of 7 units, representing 14.3%, n.s.). This blocked CMi unit displayed more ADS_{10} than the 6 nonblocked counterparts (Fig. 3E). Mechano-sensitive nociceptors also revealed higher ADS for

blocking units in control skin and after NGF treatment (Fig. 3E, gray symbols). All 15 sympathetic efferent fibers recorded from naïve animals conducted without failure at 2-Hz stimulation. After NGF treatment, 2 sympathetic efferents blocked conduction and displayed larger ADS ($P < 0.001$; ANOVA, data not shown).

4. Discussion

In the present study, we observed maximum electrically induced hyperalgesia 3 weeks after intradermal NGF administration. This time point of hyperalgesia development parallels the mechanical hypersensitivity reported recently upon NGF injection [39]. Thus, a mechanistic link between electrically and mechanically induced hyperalgesia may be suggested. Apart from threshold reduction, hyperalgesia develops when a similar primary depolarization induces higher frequencies of action potential discharges and their unimpaired axonal propagation. In order to investigate whether NGF modulates C fibers' ability to conduct high-frequency input, electrical stimuli were delivered transdermally at different frequencies, causing direct excitation of the axons and avoiding the stimulation of sensory endings. It has been shown in humans that changes of stimulation frequency modulate pain intensity, axon reflex vasodilation, and axon reflex-mediated sweating (QSART) [12,42]. Here, we provide evidence that NGF exclusively increases frequency-dependent pain, but does not affect sweat output. Employing electrophysiological recordings from the pig saphenous nerve, we further show that NGF dramatically alters the axonal action potential conveyance of unmyelinated nociceptors without producing a similar change in the axonal characteristics of sympathetic efferents. It is suggested that such differential NGF effects on axonal properties of unmyelinated fibers underlie the functional differences observed in human skin.

4.1. NGF and electrically induced nociceptor activation in human

Pain evoked by electrical stimulation increased at the NGF-treated sites when compared to NaCl control skin. Central sensitization processes following treatment with NGF, such as increased brain-derived neurotrophic factor release and sensitization of dorsal horn neurons [32], cannot be completely ruled out for an explanation (for review, see [37]). However, NGF-induced hyperalgesia was confined to the injection site and did not spread into the uninjured surrounding skin [39], indicating conditions of primary hyperalgesia. A decrease of nociceptor activation thresholds or facilitated high-frequency action potential conduction upon suprathreshold nociceptor activation may cause primary hyperalgesia. In a previous study, exploring the NGF effects in human skin, we found no evidence for reduced electrical pain thresholds, at least for currents up to 5 mA, and no alteration of electrically induced activation of mechano-insensitive C fibers as assessed by unchanged intensity-dependent axon reflex erythema [39]. In addition, perception thresholds to electrical stimuli were not affected by NGF. These data suggest that the observed electrical hyperalgesia is not based on a threshold reduction but on frequency-dependent effects.

C nociceptors can follow trains of pulses given at low frequencies of 1–4 Hz, and partially also of 5 Hz [41,46]. In contrast to Aδ fibers, most C fibers cannot follow frequencies of 20 and 100 Hz for prolonged periods [54], although peak discharge frequencies >150 Hz can be conducted for a few impulses [49]. Increased electrically evoked pain at the NGF-treated skin sites exposed at 5, 20, or 100 Hz could therefore be explained by an axonal sensitization allowing the C nociceptors to convey higher stimulation frequencies for longer periods. NGF-evoked sensitization of Aδ fibers underlying the enhanced pain responses appears unlikely, as Aδ fibers can conduct 5-Hz stimuli already under normal conditions. We therefore assume an NGF-evoked axonal sensitization of C nociceptors underlying hyperalgesia at this frequency. Thus, NGF effects on the C fibers' ability to follow high-frequency (2 Hz) input were assessed electrophysiologically in pig skin.

4.2. NGF and electrically induced nociceptor activation in pig

Repetitive stimulation of nerve fibers, particularly C nociceptors, increases their activation thresholds and slows down their action potential conduction (ADS). The levels of ADS might predict fibers' ability to generate high-frequency discharges, as suggested recently by a study in the rat saphenous nerve, demonstrating that the degree of activity-dependent slowing correlates negatively to the peak discharge rates [44]. In our study, porcine C fibers were stimulated for 3 minutes at 2 Hz. If stimulation intensities were only 1.5 times the electrical threshold of the recorded unit, a conduction failure was present in about 65% of C fibers. In this case, we individually adjusted the stimulus intensity to the minimal value (''relative stimulus strength'') required to ensure that the nerve fibers follow the 2-Hz stimulation for 3 minutes. In accordance with Taguchi et al. [44], we found that ADS positively correlates to an increased probability of conduction failure.

In humans and pigs, C nociceptors separate into mechano-insensitive (''CMi'' or ''silent'') and mechano-sensitive (''CM'' or ''polymodal'') units, based on their different amount of ADS (with CMi >> CM) [35,50]. Here, we found that CMi nociceptors maintained conduction at 2 Hz for 3 minutes only, if they were stimulated with more than twice the current intensities than those needed for CM nociceptors or sympathetic efferents, indicating a propensity of CMi nociceptors for conduction failure at high-frequency stimulation. Moreover, CMi nociceptors revealing conduction failure at 2 Hz despite augmented stimulus intensities have more pronounced ADS than the nonblocking counterparts. These findings correspond to previous microneurography studies in humans, showing that C fibers with high ADS reveal early conduction block when stimulated at high frequencies [41]. It is considered that high ADS in nociceptors [9] derives from an enhanced use-dependent slow inactivation of the nociceptor-specific sodium channel isoform Nav1.8 [6,23]. Thus, CMi nociceptors exhibiting the highest ADS may express more Nav1.8 than CM units, and therefore are more prone to conduction failures at higher discharge frequencies.

Following NGF treatment, both relative stimulation intensity and ADS were considerably reduced in CMi nociceptors, along with a reduced incidence of conduction failure. These results indicate facilitated axonal excitation (ie, reduced stimulus intensity required) in conjunction with an increased ability to follow higher input frequencies (ie, reduced ADS), both of which may contribute to the increased pain ratings upon electrical stimulation recorded herein in humans. Axonal sensitization of nociceptors might derive from an NGF-evoked alteration in the expression of voltage-dependent sodium channels, for example, Nav1.7 [18] and/or Nav1.8 [36]. Increased Nav1.7 activity in nociceptors expressing Nav1.8 would facilitate conduction during activity-dependent hyperpolarization [20]. Regardless of the underlying mechanism, NGF-induced axonal sensitization of CMi nociceptors obviously cannot account for the NGF-evoked mechanical hyperalgesia reported previously in humans [39] unless these CMi units had been sensitized to mechanical stimuli by NGF. Given that the ADS pattern of CMi nociceptors has changed also upon NGF treatment, we have at present no measures to differentiate these sensitized CMi units from genuine CM fibers.

4.3. NGF-effects on sympathetic efferents

NGF receptors are present on postganglionic efferents, and a retrograde transport with upregulated protein transcription rates

[33] as well as increased terminal sprouting of sympathetic neurons [2,17] has been documented. Furthermore, NGF enhances sodium currents [15] independently of their activation or inactivation kinetics [25], maintains calcium currents, decreases potassium current amplitude [27], and enhances discharge frequencies [24] in sympathetic neurons. Nevertheless, in our study, NGF had no effect on the activity-dependent slowing of sympathetic fibers in the pig. Noteworthy, sympathetic neurons have a different distribution pattern of voltage-dependent sodium channels compared to sensory afferent units. Nav1.6 is expressed at high levels in sympathetic, but not in C-afferent fibers [40] and can sustain high-frequency firing [20]. However, its expression is not controlled by NGF [16]. Differentially, a lack of Nav1.8 has been demonstrated in mammalian sympathetic ganglia [1,40] and might explain their low ADS. Moreover, the NGF-induced upregulation of Nav1.7 expression in sympathetic neurons [45] might generate effects contrary to those on NaV1.8-expressing nociceptors. Depending on the presence of the Nav1.8 isoform, mutated Nav1.7 channels induced hyperexcitability in sensory neurons, but resulted in hypoexcitability in sympathetic neurons [40]. Indeed, we recorded 2 sympathetic units with conduction failure, indicating hypoexcitability after NGF treatment.

Confirming our data from the pig, we found that NGF indeed does not enhance electrically evoked sudomotor function in human skin. NGF promotes the survival of noradrenergic, but not cholinergic sympathetic neurons in vitro [4], and it increases sympathetic p75-receptor expression that might sequester NGF from its high-affinity trkA receptor [33]. Furthermore, a surplus of epidermal NGF inhibited the normal sympathetic innervation of the vasculature and sweat glands, while aberrant sympathetic fibers were distributed in a dermal plexus together with trkA-expressing sensory fibers [7,8,19], suggesting their NGF-promoted functional interaction.

In summary, intracutaneous NGF sensitized nociceptors' responses to higher frequency stimulation, most probably due to axonal sensitization. In contrast, although trkA positive, we found no evidence for either functional or axonal sensitization of sympathetic efferents. The specificity of NGF to sensitize nociceptors, but not sudomotors, can be linked to differences in sodium channel distribution between nociceptors and sympathetic efferents.

Conflict of interest statement

There are no conflicts of interest to be declared.

Acknowledgements

We thank Ilona Rossbach for her editorial support and Elmar Forsch for his technical assistance. This work was supported by the Kompetenzzentrum Schmerz, State Baden Wuerttemberg (Germany), International Association for the Study of Pain, AstraZeneca R & D, Södertälje, Sweden, and the Scan|Design foundation.

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