Basic Science
Research Paper

OPEN

PAIN REPORTS

Sustained nerve growth factor-induced C-nociceptor sensitization to electrical sinusoidal stimulation in humans

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Abstract

Introduction: Injection of recombinant human nerve growth factor (rhNGF) evokes acute heat and prolonged "polymodal" (mechanosensitive [CM]) and "silent" (mechano*in*sensitive [CMi]) C-nociceptor sensitization. Both nociceptor classes can be activated differentially using slowly depolarizing electrical sinusoidal stimuli.

Objectives: To explore the temporal profile of nociceptor sensitization to heat and mechanical and electrical stimuli in humans after rhNGF.

Methods: Recombinant human nerve growth factor (1 μg) and NaCl (0.9%) was injected into human forearm skin (n = 9, 50 μL/injection). Pain ratings (numeric rating scale) to transcutaneous electrical stimuli (1 ms 20 Hz rectangular pulses, 500-ms half-period sine wave [1 Hz] and 4 Hz sine wave pulses [2.5 and 60 seconds]) were assessed at days 3, 21, and 49 after injection, in addition to heat pain thresholds (HPTs, 9 × 9 mm thermode) and mechanical impact pain (4 and 8 m/second).

Results: Suprathreshold sinusoidal stimulation for specific CM (1 Hz) and combined CM and CMi (4 Hz) activation resulted in enhanced pain from day 3 post rhNGF and lasted throughout 7 weeks. These temporal dynamics contrasted minimum HPTs at day 3 (normalized by day 49) or mechanical impact pain (developing slowly until day 21 before declining depending on stimulus intensity). Correlation analyses of electrical pain indicated diverging kinetics when assessed for CM with or without concomitant CMi activation at days 3 and 21, which converged 7 weeks post rhNGF.

Conclusions: Exceptionally long sensitization of CM and CMi nociceptors by rhNGF, uncovered by suprathreshold electrical sinusoidal stimulation, indicates a signal transduction–independent long-lasting hyperexcitability of C-nociceptors that clinically may contribute to rhNGF-maintained chronic inflammatory pain.

Keywords: Hyperalgesia, Heat transduction, Inflammation, Polymodal nociceptor

1. Introduction

An increased abundance of nerve growth factor (NGF) has been associated with chronic inflammatory pain states like osteoarthritis or pancreatitis.[34,37] A causal relationship between elevated NGF levels and chronic inflammatory pain was provided by late-phase clinical trials with NGF-neutralizing antibodies showing analgesic efficacy in patients.[14,30,33,36] In support of anti-NGF analgesic efficacy, injection of NGF into human skin and muscle evoked prolonged hyperalgesia and myalgia.[2,5,6,17,26] The temporal profiles of, for example, heat vs mechanical hyperalgesia in healthy human skin differed dramatically,[1,6,26,38] suggesting different underlying mechanisms of thermal and mechanical hyperexcitability evoked by NGF. Mechanistically, animal research revealed that NGF enhanced nociceptive transduction through phosphorylation of, for example, TRPV1,[7] generated increased currents of, for example, voltage-gated sodium channels,[9,35] increased axonal arborization ("branching") of the terminal nociceptor tree,[11,40] or even altered spinal pain signaling (eg, through brain-derived

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Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's Web site (www.painrpts.com).

PR9 9 (2024) e1190

http://dx.doi.org/10.1097/PR9.0000000000001190

neurotrophic factor [BDNF]).[15,16] In human experimental models, latter central sensitization-like changes mediated by NGF were not detected,[26] although they cannot be excluded by psychophysical measurements that only indirectly measure central sensitization based on secondary hyperalgesia.

In the present explorative human psychophysical study, therefore, we assessed the temporal sensitization profile of heat transduction and mechanical impact stimulation in parallel to neuronal excitability patterns upon rectangular and slowly depolarizing sinusoidal electrical current profiles circumventing sensory transduction. The electrical stimulation comprised profiles of 0.5-second sinusoidal pulses (1 Hz) of maximum 1 mA amplitude that induce a burst of action potentials (APs) in mechanical and heat sensitive "polymodal" C-nociceptors (CM)[27] and 4 Hz sinusoidal cycles of maximum 0.4 mA amplitude that evoke a single AP per cycle in both polymodal and "silent" (mechanical-*in*sensitive) C-nociceptors (CMi).[12] Differential activation of C-nociceptors thereby may indicate the contribution and temporal dynamics of recombinant human nerve growth factor (rhNGF)–induced "polymodal" CM and "silent" CMi nociceptor excitability changes. Correlation analyses of pain ratings to electrical 1 Hz and 4 Hz sinusoidal stimulation uncovered that sensitization mechanisms and their afferent substrates vary at early time points but converge on a unified electrical C-nociceptor sensitization 7 weeks after NGF.

2. Methods

The Ethic Committee II of the Medical Faculty Mannheim at the University of Heidelberg approved the study protocol and experimental procedures (reference 2017-644N-MA). All volunteers signed a written consent form at least 24 hours before study onset. Subjects were familiarized with the electrical and mechanical impact stimulation during an information session, thereby practicing the use of the numeric rating scale (NRS) with the end points 0 (no pain) and 10 (worst pain imagined) for magnitude pain assessment.

2.1. Subjects and recombinant human nerve growth factor injection

In total, 11 volunteers were recruited for the study, but 2 subjects had to be excluded because of SARS-CoV-2 infection occurring during the 49-day observation period. Hence, 9 subjects (5 women and 4 men, average age 39 ± 5 years) received 50 μL of intradermal injections (Becton Dickinson 30 G syringe, Heidelberg, Germany) of 1 μg recombinant human β-NGF (rhNGF, Miltenyi Biotec, Bergisch-Gladbach, Germany) dissolved in sterile 0.9% NaCl and 0.9% NaCl (vehicle) medial into their left and right volar forearm equidistant to the wrist and cubital fossa. Recombinant human nerve growth factor was freshly reconstituted directly before intradermal injection. We suspected that storage of reconstituted and frozen rhNGF can reduce or shorten rhNGF-induced hyperalgesia, similar to reduced activity previously reported for cytokines in human serum stored at −80°C.[4]

All injection sites were photographed and labelled with a felt tip pen. Labels were copied on translucent paper together with characteristic skin marks (eg, mole, birthmark, veins) for the recognition of the rhNGF/NaCl spots throughout the 49-day period.

2.2. Experimental protocol

Pain in response to electrical stimuli was investigated first, followed by mechanical impact pain assessment and finally heat pain threshold measurements, all of which were applied at both injection spots (randomized rhNGF and NaCl sites) at days 3, 21, and 49 after injection. Of note, all measures at NaCl sites remained stable over the whole observation period indicating that repetitive stimulation at frequencies used herein did not change sensitivity to the employed psychophysical tests over time.

2.3. Electrical stimulation

A bipolar blunted platinum electrode (diameter 0.4 mm, distance 2 mm, Nørresundby, Denmark) mounted on an applicator printed with a 3D-printer was attached to the rhNGF/NaCl-treated forearm skin sites.

Rectangular electrical pulses (width 0.1 ms, frequency 20 Hz) were triggered externally by a pulse generator (PG1, Rimkus Medizintechnik, Parsdorf, Germany) and delivered with increasing current intensities of 0.1 mA/second (Digitimer DS7 constant current stimulator, Welwyn Garden City, United Kingdom) until subjects reported a first sensation (perception threshold), pulses were perceived painful (pain threshold), and pulses were estimated NRS 3 intensity (suprathreshold pain).

Half-sine wave pulses of 500-millisecond duration (Digitimer DS5, Welwyn Garden City, United Kingdom) were delivered computer controlled (Dapsys 8 software©, Brian Turnquist, Minnesota, MN) by means of a Digital-Analogue Converter (NI USB-6221, National Instruments, Austin, TX) in increments of 0.2 mA and in randomized order to a maximum of 1 mA. Volunteers were instructed to rate for each pulse the maximum perceived pain on the NRS.

Sine wave pulses of 4 Hz were delivered at random intensities of 0.05, 0.1, 0.2, and 0.4 mA for 2.5 seconds each (Digitimer DS5), Dapsys 8 software (©Brian Turnquist), NI USB-6221 Digital-Analogue Converter (National Instruments), and subjects rated the maximum pain (NRS) per stimulus.

Finally, 4 Hz sine wave pulses of 0.2 mA were delivered continuously for 60 seconds. Volunteers were instructed to estimate maximum pain (NRS) at 5 and 10 seconds after stimulus onset and thereafter at 10-second intervals until the end of stimulation.

Apart from the 60-second stimulation, all electrical pulses were delivered twice, and mean values of duplicate stimulation were used for statistical analysis. Time intervals of at least 10 seconds were kept between the electrical stimulation paradigms.

2.4. Mechanical impact pain

A cylindrical plastic bullet (12-mm height, 5-mm diameter, 0.5-g weight) was computer controlled accelerated to a velocity of 4 and 8 m/second by means of compressed air and directed by an 8-cm barrel perpendicular to the rhNGF- and NaCl-treated skin surface. Each velocity was investigated 3 times, starting with 4 m/second followed by 8 m/second and with 10-second intervals in between. Pain intensity of the impact stimulus was rated by the volunteer on the NRS, and average NRS values for each velocity were calculated for statistical analysis.

2.5. Heat pain threshold

A 9 × 9 mm Peltier thermode (Somedic, Sösdala, Sweden) was attached to the rhNGF-treated/NaCl-treated skin sites. The temperature of the thermode increased computer controlled from 32°C by 1°C/second (MSA, Sense5.2, Somedic) to a cutoff of 50°C, and volunteers were instructed to press a handheld switch as soon as the temperature was perceived as painfully

hot. Thereafter, thermode temperature returned to 32°C. Heat pain thresholds were assessed 3 times with 10-second intervals, and average temperatures calculated for statistical analysis.

2.6. Correlation analyses

Parametric Pearson and nonparametric Spearman correlation coefficients were calculated on delta NRS values (rhNGF—NaCl) upon 500-millisecond half-sine wave stimuli (0.2–1 mA), 4 Hz sine wave 2.5-second pulses (0.1–0.4 mA), area under the curve (AUC) of continuous 4 Hz sine wave stimulation (60 seconds, 0.2 mA), and mechanical impact stimulation (4 m/second and 8 m/second), and on delta (NaCl—rhNGF) heat pain thresholds (HPTs) and rectangular electrical current (0.1 ms, 20 Hz) amplitudes for pain thresholds and suprathreshold pain. Correlation coefficients and *P* values associated with their significance were plotted as heat maps.

2.7. Statistics

All statistics and graphical representations were performed using Prism 8.4.3 (GraphPad, San Diego, CA) and R 4.2.2 (https://cran.r-project.org/bin/windows/base/) package rstatix 0.7.2 (https://rdrr.io/r/utils/install.packages.html). For electrical and mechanical stimuli, parametric 3-way repeated-measures (RM) analysis of variance (ANOVA) was calculated between the factorial groups "rhNGF"—"day"—"stimulus intensity" ("rhNGF"—"day"—"time" for 1 min 4 Hz sine wave stimulation). Identified interactions within the factor rhNGF and intensity (respectively "time" for 1 minute 4 Hz sine wave) were reanalyzed using 2-way RM-ANOVA with the grouping variable "day," followed by Bonferroni multiple comparison post hoc test. Similarly, HPTs were analyzed by 2-way ANOVA (factorial groups rhNGF—day) and Bonferroni comparison. Sphericity was confirmed by visual QQ-plot inspection and Geisser-Greenhouse correction if necessary.

Delta NRS values (or delta AUC values for continuous 60-second sinusoidal stimulation) between rhNGF and NaCl were analyzed nonparametrically and deviations of delta values from a theoretical value of zero (= no effect of rhNGF) assessed using one-sample Wilcoxon matched pairs tests. Significant differences between experimental days were assessed with Friedman 1-way ANOVA and Dunn post hoc test.

Data analyzed with parametric tests are presented as means ± SEM. Data analyzed with nonparametric tests are given as median ± interquartile range. *P* values <0.05 were considered significant and indicated in the figures with appropriate signs (#: RM-ANOVA, +: Bonferroni post hoc test, *: Wilcoxon matched pairs test, $: Dunn test).

3. Results

We used psychophysics to compare responses to various somatosensory stimuli at both rhNGF and 0.9% NaCl injection sites over an observation period of 49 days.

3.1. Heat pain threshold

Signal transduction at nociceptive terminals was assessed by HPT measures. Heat pain thresholds changed significantly at the rhNGF injection site over time ($F(2,16) = 17.87$, $P < 0.0001$, 2-way RM-ANOVA, **Fig. 1A**). They reached their maximum reduction at day 3 (rhNGF: 43.7°C vs NaCl: 48.0°C) and normalized until day 49 (day 21: 46.9°C, day 49: 48.9°C). Similar to our parametric analysis, nonparametric data evaluation of HPTs showed significant site differences early after rhNGF treatment (day 3 and 21, **Fig. 1B**) that normalized over 49 days. These results indicated an acute but transient sensitization for heat transduction driven by rhNGF.

3.2. Mechanical impact pain

At suprathreshold intensities, mechanical impact stimuli induce a volley of action potentials in mechanosensitive C-nociceptors after corresponding transduction processes. Recombinant human nerve growth factor induced a significant hyperalgesia to mechanical impact stimuli ($F(1,8) = 48.16$, $P < 0.0002$, 3-way RM-ANOVA) that interacted with the day of assessment ($F(2,16) = 4.39$, $P < 0.05$, 3-way RM-ANOVA) and stimulus intensity ($F(1,8) = 9.51$, $P < 0.02$ 3-way RM-ANOVA) with strong combined rhNGF, time, and intensity effects ($F(2,16) = 3.92$, $P < 0.05$, 3-way RM-ANOVA). At low stimulus intensity (4 m/second), mechanical impact pain was significantly elevated at rhNGF sites particularly at day 21 (increase in NRS 1.7 ± 0.4, **Fig. 2A**). High mechanical impact stimulation (8 m/second) was also perceived stronger after rhNGF and remained elevated, although not significantly after 49 days (NRS 2.5 ± 0.3 vs NRS 1.8 ± 0.2 at the NaCl site, **Fig. 2B**). Accordingly, delta NRS values between rhNGF and NaCl sites to mechanical impact stimuli were strongest at day 21 for both 4 m/second (**Figs. 2C**) and 8 m/second (**Fig. 2D**) and declined afterward. Based on these results, a facilitated and particularly sustained increase of neuronal excitability along with enhanced signal transduction may be suggested upon suprathreshold high impact stimulation of mechanosensitive C-nociceptors after rhNGF challenge.

3.3. Sensory thresholds for rectangular electrical currents

Transcutaneous rectangular electrical stimuli of 0.1-millisecond pulse width and 20 Hz activate myelinated Aβ-fibers and with increasing current amplitudes thinly myelinated Aδ-fibers and eventually C-nociceptors. Recombinant human nerve growth factor did not significantly alter transcutaneous rectangular electrical current amplitudes required to elicit sensations (n.s., **Fig. 3A**). Currents for pain thresholds ($F(1,8) = 7.52$, $P < 0.03$, 2-way RM-ANOVA, **Fig. 3B**) and NRS 3 pain ($F(1,8) = 21.29$, $P < 0.002$, 2-way RM-ANOVA, **Fig. 3C**) were significantly lower compared with NaCl across all assessment days, particularly for NRS 3 pain on day 21 (rhNGF 2.2 ± 0.3 mA vs NaCl 3.4 ± 0.4 mA). Nonparametric analysis of delta current amplitudes between NaCl- and rhNGF-treated sites indicated that rhNGF treatment reduced the current amplitudes required to evoke painful but not innocuous sensations (**Fig. 3D–F**). The results indicate persistent sensitization of thinly myelinated Aδ-nociceptors and unmyelinated C-nociceptors by rhNGF, with C-nociceptors displaying a particularly sustained hyperexcitability, while myelinated low-threshold Aβ-fibers appeared not affected.

3.4. Sensation to 500-milliseconds half-sine wave stimulation

To specifically investigate neuronal excitability of C-nociceptors bypassing transduction mechanisms, we applied electrical single half-period 1 Hz sinusoidal pulses (duration 500 ms) that induce a burst of APs in mechanosensitive C-nociceptors. Half-sine wave pulses of 0.2–1 mA evoked intensity-dependent pain ($F(4,32) = 116.08$, $P < 0.00001$, 3-way RM-ANOVA) that was perceived significantly stronger at the rhNGF site ($F(1,8) = 22.45$, $P < 0.002$, 3-way RM-ANOVA) across all days of assessment

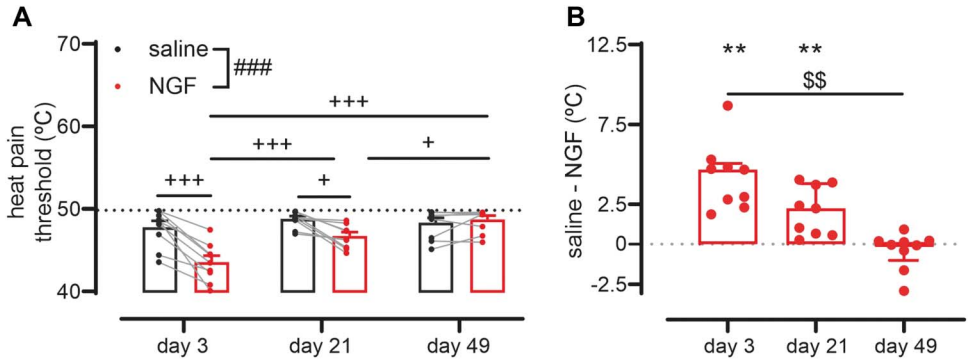

Figure 1. rhNGF-mediated rapid HPT reduction is transient. HPTs were measured in NaCl-injected or rhNGF (1 μg)-injected forearm skin of 9 subjects over 49 days. (A) Parametric analysis revealed a rapid decline in HPTs after rhNGF treatment at day 3 and day 21 ($P < 0.0001$ and $P < 0.05$, Bonferroni multiple comparison test) that normalized completely over the 49-day observation period (n.s., Bonferroni multiple comparison test). The dashed line indicates the cutoff temperature of 50°C of the thermode. Individual data points and means ± SEM are given. ###$P < 0.0005$ (2-way RM-ANOVA) with Bonferroni multiple comparison tests (+$P < 0.05$, +++ $P < 0.0005$). (B) Nonparametric analysis of net rhNGF effects (delta values) corroborated the rhNGF-induced HPT decline (NaCl vs rhNGF at day 3 and day 21: ** $P < 0.004$ and at day 49: $P > 0.7$, Wilcoxon matched pairs tests) with rapid onset but only transient existence ($F = 12.67$, $P < 0.001$, Friedman ANOVA; day 3 vs day 49: $$ $P < 0.002$, Dunn post hoc test). Individual data points and median ± IQR are given. HPT, heat pain threshold; IQR, interquartile range; rhNGF, recombinant human nerve growth factor.

(rhNGF–intensity interaction $F(4,32) = 4.80$, $P < 0.004$, 3-way RM-ANOVA). Differences of pain magnitude between rhNGF-treated and NaCl-treated sites were present already for 0.2 mA (**Fig. 4A**) and 0.4 mA (**Fig. 4B**) at day 21. Stronger stimulation of 0.6 mA (**Fig. 4C**) and strongest half-sine wave pulses of 0.8 and 1.0 mA (**Fig. 4D, E**) resulted in significantly higher pain ratings at rhNGF-injected sites lasting for 7 weeks (eg, 1 mA NRS 3.4 ± 0.4 [rhNGF] vs 2.4 ± 0.2 [NaCl]). Nonparametric analysis of cumulative rhNGF effects (delta NRS) across all half-sine current intensities corroborated significantly augmented pain above zero at the rhNGF sites throughout the complete 49-day period (**Fig. 4F**). In accord to high mechanical impact pain stimulation, this result indicated a sustained hypersensitivity of mechanosensitive C-nociceptors after rhNGF.

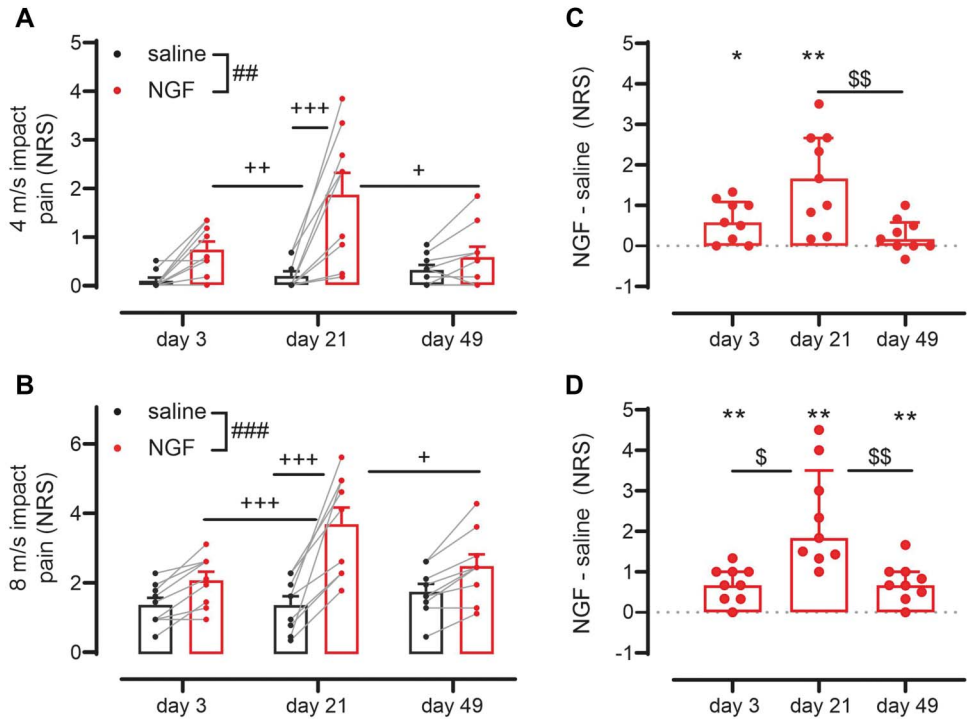

Figure 2. rhNGF produces slow-onset but long-lasting hyperexcitability to mechanical impact stimulation. Mechanical impact pain (4 m/second (A) or 8 m/second (B)) revealed rhNGF-induced hyperexcitability ($F(1,8) = 48.16$, $P < 0.0002$, 3-way RM-ANOVA) that was present across all assessment days (interaction rhNGF × day $F(2,16) = 11.54$, $P < 0.005$, 3-way RM-ANOVA). Hyperalgesia peaked at 21 days for both 4 m/second ($P < 0.05$, Bonferroni multiple comparison test) and 8 m/second ($P < 0.00005$, Bonferroni multiple comparison test). Pain upon high mechanical impact (8 m/second) remained elevated at day 49, however not significant ($P = 0.25$, Bonferroni multiple comparison test). Individual data points and means ± SEM are given. ##$P < 0.005$, ###$P < 0.0005$ (2-way RM-ANOVA) with Bonferroni multiple comparison tests (+$P < 0.05$, ++ $P < 0.005$, +++ $P < 0.0005$). Nonparametric analysis of net rhNGF effects (delta values) corroborated the overall time course of rhNGF-induced mechanical hypersensitivity ((C) 4 m/second: $F = 12.67$, $P < 0.001$; (D) 8 m/second: $F = 12.51$, $P < 0.001$; Friedman ANOVA) and the impact intensity–dependent hyperexcitability at day 49 post rhNGF (8 m/second: $P < 0.01$; Wilcoxon matched pairs tests). Individual data points and median ± IQR are given. */$ $P < 0.05$, **/$$ $P < 0.005$, IQR, interquartile range; rhNGF, recombinant human nerve growth factor.

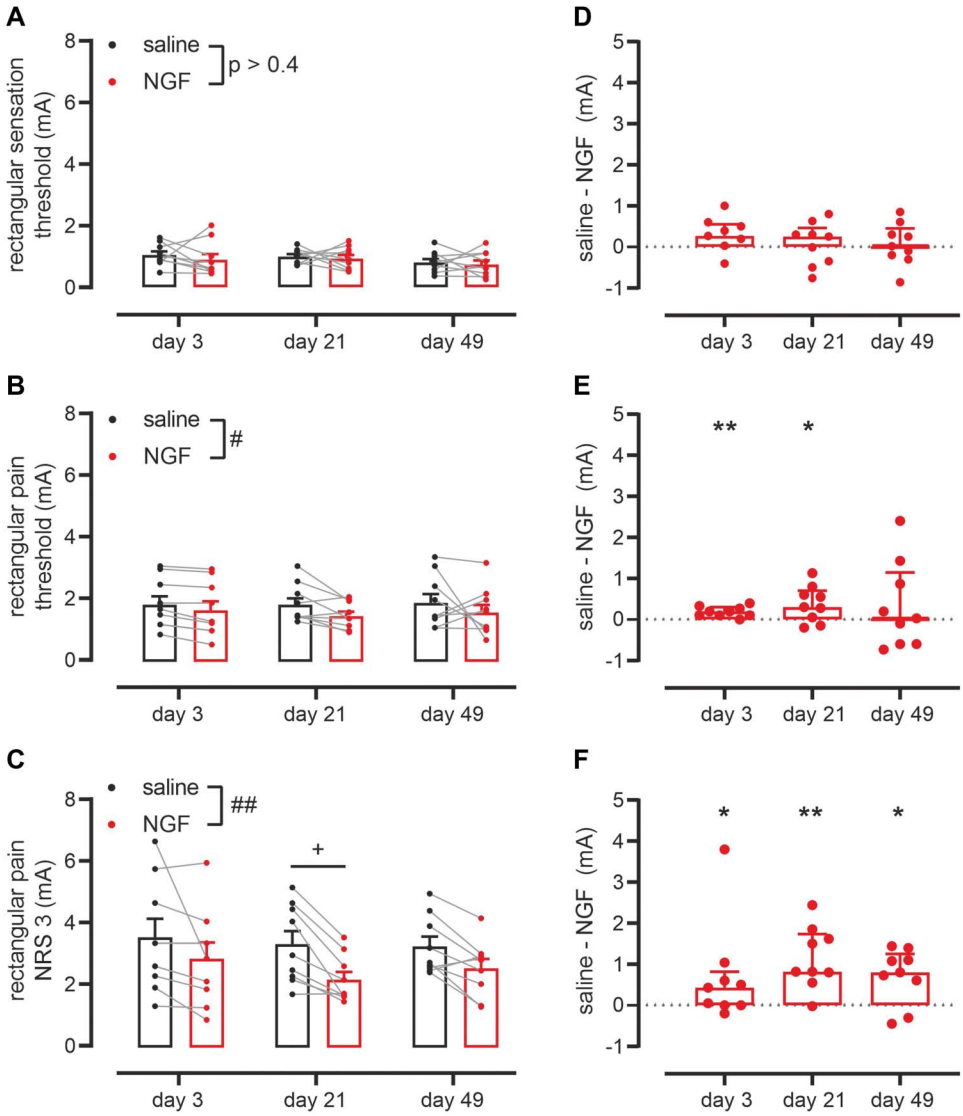

Figure 3. rhNGF decreases rectangular electrical currents necessary to induce painful sensations. Transcutaneously delivered rectangular electrical current amplitudes (0.1 ms, 20 Hz) necessary to elicit any (innocuous) sensation (A), a painful sensation (B), or pain NRS 3 (C) were measured in NaCl-injected or rhNGF (1 μg)-injected forearm skin of 9 subjects over 49 days. (A–C) Parametric analysis revealed that rhNGF only sensitizes for rectangular electrical current stimulation in the painful range (A: $F(1,8) = 0.58$, $P > 0.4$; B: $F(1,8) = 7.52$, $P < 0.03$; C: $F(1.8) = 21.29$, $P < 0.002$; 2-way RM-ANOVA). No interaction of sensitization to rectangular stimulation and its time course was recorded for pain threshold (B, $F(2,16) = 0.16$, $P > 0.8$, ANOVA) and pain NRS 3 (C, $F(2,16) = 0.76$, $P > 0.48$, ANOVA). Individual data points and means ± SEM are given with #$P < 0.05$ and ##$P < 0.005$ (2-way RM-ANOVA) and Bonferroni multiple comparison tests (+$P < 0.05$). (D–F) Nonparametric analysis of net rhNGF effects (delta values) corroborated that rhNGF does not sensitize for innocuous sensations (D, day 3: $P > 0.32$, day 21: $P > 0.79$, day 49: $P > 0.75$) but for pain thresholds (E, day 3: $P < 0.01$, day 21: $P < 0.04$, day 49: $P > 0.55$) and pain NRS 3 (F, day 3 $P < 0.05$, day 21 $P < 0.01$, day 49 $P < 0.02$; Wilcoxon matched pairs tests). Individual data points and median ± IQR are given. IQR, interquartile range; NRS, numeric rating scale; rhNGF, recombinant human nerve growth factor.

3.5. Sensation to 2.5-second 4 Hz sinusoidal stimulation

We also assessed the activation patterns of mechano*in*sensitive C-fibers, another subclass of C nociceptors that can be activated along with mechanosensitive C-fibers by 4 Hz sinusoidal electrical stimulation. Sinusoidal pulses of 4 Hz delivered for 2.5 seconds evoked an intensity-dependent increase in pain ($F(3,24) = 244,8$, $P < 0.0001$, 3-way RM-ANOVA) at the rhNGF injection site ($F(1.8) = 14.15$, $P < 0.01$, 3-way RM-ANOVA) with strong "rhNGF"—"current intensity" interaction ($F(3,24) = 17.36$, $P < 0.00001$, 3-way RM-ANOVA) across all days of assessment. In detail, "low-intensity" sinusoidal stimuli of 0.05 mA were not perceived stronger at the rhNGF site (**Fig. 5A**), whereas pain upon 0.1 mA amplitudes was significantly elevated (**Fig. 5B**). Pain intensity culminated in response to "high-intensity" sinusoidal stimuli of 0.2 mA (**Figs. 5C**) and 0.4 mA (**Fig. 5D**) and hypersensitivity to them persisted until day 49 (rhNGF 0.2 mA NRS 2.4 ± 0.4 vs NaCl 1.6 ± 0.3 ($P < 0.05$) and rhNGF 0.4 mA NRS 4.2 ± 0.5 vs NaCl 2.7 ± 0.4 ($P < 0.0005$; Bonferroni multiple comparison tests). We also calculated cumulative rhNGF effects (delta NRS) for nonparametric analysis separately at "low-stimulation intensity" (0.05 and 0.1 mA, **Fig. 5E**) and "high-stimulation intensity" (0.2 and 0.4 mA, **Fig. 5F**) and corroborate significantly augmented pain above delta zero at the rhNGF sites throughout day 49 for high-stimulation intensity only. The results of 4 Hz sinusoidal electrical stimulation that additionally activates mechano*in*sensitive C-nociceptors confirmed the acute and long-lasting effect of rhNGF to enhance neuronal excitability of unmyelinated afferents.

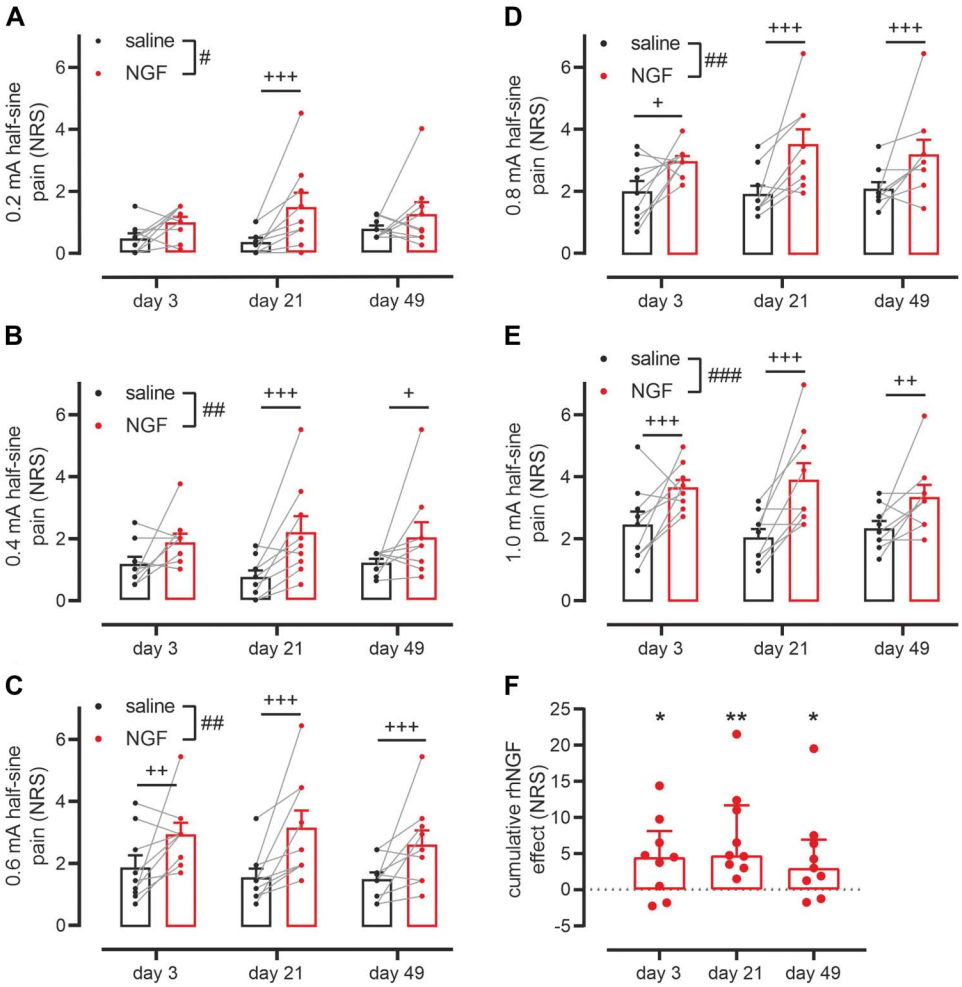

Figure 4. rhNGF increases pain induced by half-sine wave electrical stimulation. Pain elicited by transcutaneous half-sine wave electrical stimulation (500 ms, 0.2–1 mA) was measured in NaCl-injected or rhNGF (1 μg)-injected forearm skin of 9 subjects over 49 days. (A–E) Parametric analysis revealed a robust sensitization by rhNGF at all stimulation intensities (0.2 mA: $F(1,8) = 10.64$, $P < 0.02$; 0.4 mA: $F(1,8) = 14.89$, $P < 0.005$; 0.6 mA: $F(1,8) = 20.47$, $P < 0.002$; 0.8 mA: $F(1,8) = 18.13$, $P < 0.003$; 1.0 mA: $F(1,8) = 31.82$, $P < 0.0005$; 2-way RM-ANOVA), which was particularly strong at day 21 (Bonferroni multiple comparison tests, $+++ P < 0.0005$) and remained significantly elevated at day 49 even for 0.4 mA amplitudes (Bonferroni multiple comparison tests, $+ P < 0.05$). Individual data points and means ± SEM are given. $\#P < 0.05$, $\#\#P < 0.005$, $\#\#\#P < 0.0005$ (2-way RM-ANOVA) with Bonferroni multiple comparison tests ($+P < 0.05$, $++ P < 0.005$, $+++ P < 0.0005$). (F) Nonparametric analysis of the cumulative net rhNGF effect across all half-sine wave stimulation intensities corroborated the robust and long-lasting hyperexcitability induced by rhNGF even at day 49 (day 3: $P < 0.05$; day 21: $P < 0.01$; day 49: $P < 0.05$; Wilcoxon matched pairs tests). Individual data points and median ± IQR are given. * $P < 0.05$, ** $P < 0.005$. IQR, interquartile range; rhNGF, recombinant human nerve growth factor.

3.6. Continuous 1 minute 4 Hz sinusoidal 0.2 mA stimulation

We finally explored whether the known pronounced accommodation of unmyelinated C-nociceptor excitation upon sustained 4 Hz sinusoidal stimulation had changed after exposure to rhNGF. Pain intensity reported during 60 seconds of 4 Hz stimulation (0.2 mA) was significantly different between rhNGF-treated and NaCl-treated sites ($F(1,8) = 9.15$, $P < 0.02$, 3-way RM-ANOVA). Hypersensitivity at the rhNGF site was particularly prominent on day 3 ($F(1,8) = 23.07$, $P < 0.002$, 2-way RM-ANOVA, **Fig. 6A**) and less pronounced on day 21 ($F(1,8) = 2.76$, $P > 0.13$, 2-way RM-ANOVA, **Fig. 6B**) and day 49 ($F(1,8) = 2.59$, $P > 0.14$, 2-way RM-ANOVA, **Fig. 6C**). Noteworthy, intracutaneous rhNGF delivery did not affect pain accommodation typically recorded in normal skin to sustained sine wave electrical stimulation at any assessment day (interaction rhNGF × seconds $F(6,48) = 1.01$, $P > 0.42$, 3-way RM-ANOVA). For nonparametrical analysis, we further integrated pain ratings across the 60-second stimulation period (AUC) and calculated net rhNGF effects (delta NRS, rhNGF—NaCl, **Fig. 6D**) and corroborate significantly augmented pain above zero at the rhNGF site only at day 3. Therefore, this result confirmed an enhanced sinusoidal C-nociceptor excitation during continuous stimulation, whereas the accommodation pattern remained unaltered.

3.7. Data correlation analyses

To identify overlapping sensitization mechanisms for the assessed stimulation profiles and their temporal dynamics, we performed parametric Pearson correlation on the delta values calculated between the NGF and NaCl sites for days 3, 21, and 49, respectively (**Fig. 7A**). On days 3 and 21, strong and significant correlations were detected for current intensities of half-sine wave and 4 Hz sinusoidal stimulation. By contrast, stimulus intensity–dependent correlations were found for mechanical impact at day 21 and for rectangular current amplitudes at day 3 only. When comparing rhNGF-induced hyperalgesia between the 2 electrical sinusoidal stimulation profiles, no correlation was identified for days 3 and 21 across all stimulation

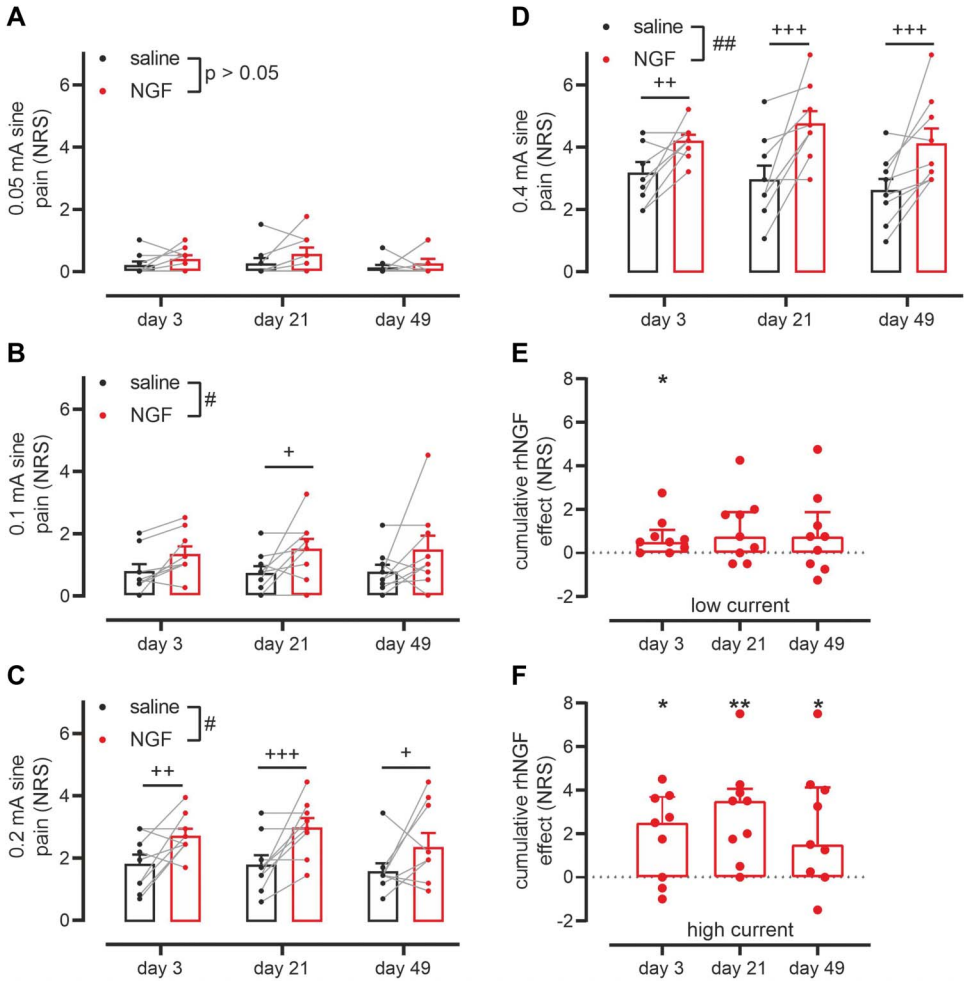

Figure 5. rhNGF increases pain induced by high-intensity 2.5-second sinusoidal electrical stimulation. Pain elicited by transcutaneous sinusoidal electrical stimulation (2.5 seconds, 4 Hz, 0.05–0.4 mA) was measured in NaCl-injected or rhNGF (1 μg)-injected forearm skin of 9 subjects over 49 days. (A–D) Parametric analysis revealed no sensitization by rhNGF at a stimulation amplitude of 0.05 mA (A: $F(1,8) = 5.18$, $P > 0.05$), whereas higher current intensities evoked significantly more pain across all days of assessment (B 0.1 mA: $F(1,8) = 6.93$, $P < 0.04$; C 0.2 mA: $F(1,8) = 14.14$, $P < 0.01$; D 0.4 mA: $F(1,8) = 21.49$, $P < 0.005$; 2-way RM-ANOVA). The time course of sensitization was also depended on the stimulation intensity and revealed consistently elevated pain ratings over the whole 49-day observation period, particularly at high-current amplitudes of 0.2 mA ($P < 0.05$, Bonferroni multiple comparison test) and 0.4 mA ($P < 0.0005$, Bonferroni multiple comparison test). Individual data points and means ± SEM are given. #$P < 0.05$, ##$P < 0.005$ (2-way RM-ANOVA) with Bonferroni multiple comparison tests (+$P < 0.05$, ++ $P < 0.005$, +++ $P < 0.0005$). (E, F) Nonparametric analysis of the cumulative net rhNGF effect at low (0.05 + 0.1 mA, E) or high (0.2 + 0.4 mA, F) sine wave stimulation intensities corroborated the robust and long-lasting hyperexcitability induced by rhNGF at high amplitudes (E, day 3: $P < 0.02$; day 21: $P > 0.08$; day 49: $P > 0.25$; F, day 3: $P < 0.05$; day 21: $P < 0.01$; day 49: $P < 0.04$; Wilcoxon matched pairs tests). Individual data points and median ± IQR are given. IQR, interquartile range; rhNGF, recombinant human nerve growth factor.

amplitudes, which changed at day 49 after rhNGF. Precisely, hyperalgesia to almost all half-sine wave and short 2.5-second sinusoidal amplitudes or pain AUC upon ongoing 60-second sine wave 0.2 mA electrical stimulation correlated strongly with each other (green square, **Fig. 7B**), and this opposed to HPTs and rectangular electrical pulse magnitudes. Correlation of mechanical impact pain with sinusoidal electrical stimulation pain was only spurious (blue squares, **Fig. 7B**). In addition, we found a strong correlation of both sinusoidal stimulation profiles at day 49 with half-sine wave stimulation at day 21 (yellow squares, **Fig. 7B**). By calculating nonparametric Spearman correlation coefficients, we got largely similar results (Supplemental Figure S1, http://links.lww.com/PR9/A247). This result suggests that temporal sensitization patterns differed between mechanosensitive and mechano*in*sensitive C-nociceptors at early time points but converged on a unified sensitization mechanism 7 weeks after rhNGF challenge.

4. Discussion

After a single intradermal injection of rhNGF into human skin, we investigated the temporal dynamics of hyperalgesia to various cutaneous stimuli within a 7-week observation period. We confirmed the development of reduced HPTs and hyperalgesia to low-intensity mechanical stimuli, which normalized completely 7 weeks post rhNGF, whereas strong mechanical stimuli were still perceived as more painful.[6,26,28,38] Suprathreshold electrical stimulation with classic 0.1-milliseconds 20 Hz rectangular pulses uncovered hyperalgesia particularly after 3 weeks and in accordance with enhanced pain after rectangular electrical stimulation reported before.[21,29,31,38] Therefore, our results indicate that a single rhNGF injection provokes hyperexcitable C-nociceptors for at least 7 weeks, and suprathreshold electrical sinusoidal or mechanical impact stimuli are well suited to expose the existence of this state.

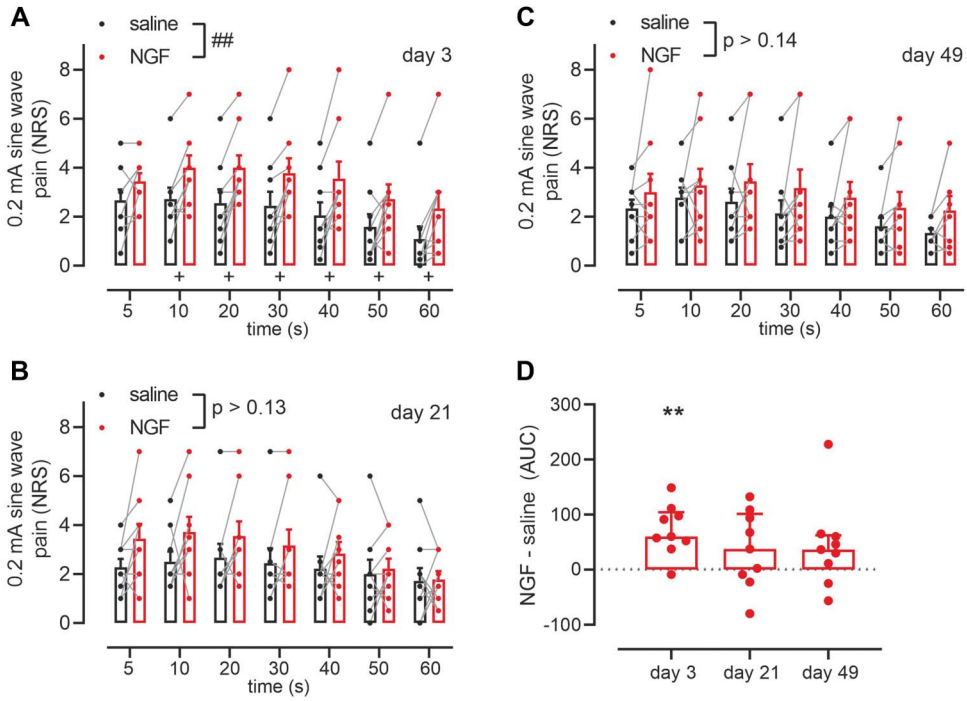

Figure 6. rhNGF does not alter adaptation to 60-second sinusoidal electrical stimulation. Pain elicited by transcutaneous sine wave electrical stimulation (60 seconds, 4 Hz, 0.2 mA) was measured in NaCl-injected or rhNGF (1 μg)-injected forearm skin of 9 subjects over 49 days. (A–C) Parametric analysis revealed a significant sensitization by rhNGF at day 3 (A, $F(1,8) = 23.07$, $P < 0.002$, 2-way RM-ANOVA) during 10-second and 60-second stimulation ($P < 0.005$, Bonferroni multiple comparison test). The trend of hyperalgesia persisted at later time points but was not significant across the 60-second stimulation period (day 21 B, $F(1,8) = 2.76$, $P > 0.13$; day 49 C, $F(1,8) = 2.59$, $P > 0.14$; 2-way RM-ANOVA). Accordingly, pain accommodation during the 60-second sinusoidal stimulation period was unchanged after rhNGF when compared with NaCl at all experimental days ($F(6,48) = 1.01$, $P > 0.42$, 2-way RM-ANOVA). Individual data points and means ± SEM are given with ##$P < 0.005$ (2-way RM-ANOVA) and Bonferroni multiple comparison tests (+$P < 0.005$). (D) Nonparametric analysis of the net rhNGF effect (delta values of the AUC) corroborated the robust but only at day 3 significant hyperexcitability induced by rhNGF (day 3: **$P < 0.008$; day 21: $P > 0.2$; day 49: $P > 0.09$, Wilcoxon matched pairs tests). Individual data points and median ± IQR are given. AUC, area under the curve; IQR, interquartile range; rhNGF, recombinant human nerve growth factor.

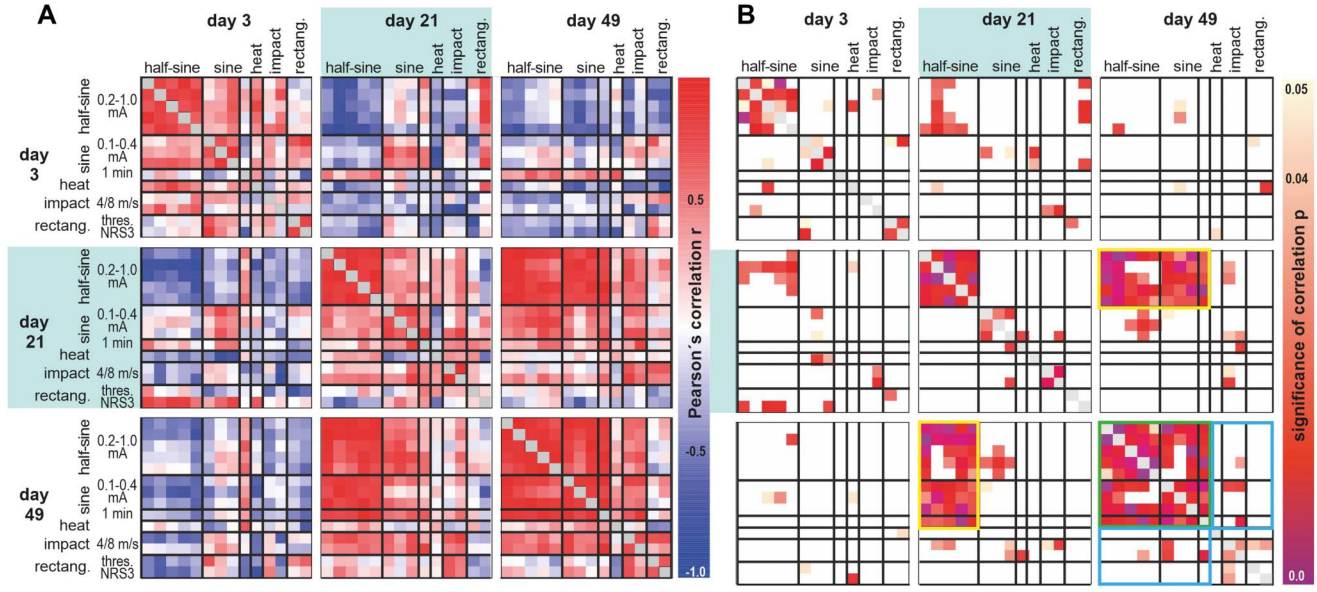

Figure 7. Increased correlation of rhNGF-induced sensitization at day 49 to different sinusoidal stimulation paradigms suggests converging mechanisms. rhNGF-dependent sensitization to the various painful stimulation paradigms at all 3 experimental days was compared using Pearson correlation. Correlation coefficients (A) and the associated significance levels (B) are plotted as heat maps. At day 3 and day 21, overlapping sensitization mechanisms are largely confined within the same stimulation paradigm. By contrast, at day 49, sensitization to half-sine wave and 2.5-second and 60-second sinusoidal electrical stimulation correlated intensively (green square), whereas correlation with mechanical impact pain and rectangular electrical current stimulation was only spurious (blue squares). Interestingly, the sensitization to half-sine and sinusoidal electrical stimulation at day 49 also correlated strongly with the sensitization to half-sine wave stimulation at day 21 (yellow squares), indicating that the latter measure can be used to predict future sensitization for all sinusoidal stimulation paradigms. rhNGF, recombinant human nerve growth factor.

4.1. C-fiber types involved in recombinant human nerve growth factor–induced hyperalgesia

Employing our slowly depolarizing sinusoidal electrical currents that activate "polymodal" CM nociceptors with (4 Hz sine wave) or without (1 Hz sine wave) concomitant recruitment of CMi nociceptors,[12,27] we aimed to dissect the temporal sensitization pattern of particular C-nociceptor subtypes after rhNGF. We found that rhNGF produced hyperalgesia to both electrical stimulation paradigms, which was maintained for the full 7-week observation period. Although single nerve fiber recordings unequivocally showed CMi sensitization after rhNGF,[8,22,23,39] our psychophysical measurements do not allow to specifically address the contribution of CMi nociceptors to rhNGF-mediated hyperalgesia. By contrast, we can conclude that responses of CM nociceptors to electrical sinusoidal stimuli are enhanced by a single rhNGF injection into human skin for at least 7 weeks. Intriguingly, correlation analyses identified a very strong correlation for 500-ms half-sine stimulation between day 21 and day 49 post rhNGF. It thus may be suggested that CM nociceptors remained hyperexcitable throughout 49 days.

4.2. Mechanisms of recombinant human nerve growth factor–induced hyperalgesia

One general finding of our study is a notable lack of correlation between hyperalgesia to electrical activation and stimulation with natural stimuli at any time point. Obviously, heat and mechanical impact stimulation require the transduction of the stimulus into a receptor potential, and this is bypassed by direct electrical stimulation. For heat pain, it is a sensitization of this transduction process that is believed to dominate rhNGF-induced hyperalgesia, for instance, through enhanced plasma membrane availability of the heat-sensitive ion channel TRPV1.[41] We speculate that the sustained mechanical hyperalgesia post rhNGF could be mediated by a similar mechanism of sensitized mechanotransduction, for instance, through the stretch-activated ion channel PIEZO2.[20,25] The lacking correlation thus indicates independent mechanisms of rhNGF-induced hyperexcitability involving the signal transduction machinery separated from the AP initiation and propagation processes after signal transformation.

Irrespective of sensory transduction, the differential response patterns to half-sine and sine wave stimulation may allow to narrow down and temporally specify the previously postulated peripheral sensitization to rectangular electrical stimulation after rhNGF.[29] However, 4 Hz sine wave stimuli induce single APs per cycle that mainly depend on passive membrane properties, in particular the time constant of the C-fiber axolemma, which is approximately 100 milliseconds in both CM and CMi nociceptors[3] approximately matching the duration of our 4 Hz sinusoidal half-cycle (125 ms), and the membrane resistance, which is increased at lower temperatures resulting in enhanced sinusoidal responses upon cooling.[24] These passive membrane properties are also governing the passive response to half-sine wave stimulation, provoking the first action potential of the half-sine-induced AP burst. Hence, an alteration of the biophysical membrane characteristics, including longer time constants (smaller axonal diameter) and higher membrane resistance (lower potassium conductance),[24] may increase the sensitivity to both depolarizing profiles. Nerve growth factor–induced structural changes include axonal growth and outgrowing nerve fiber terminals are expected to be thinner with accordingly longer membrane time constants. Assuming that such rhNGF-induced structural changes require some time to develop but last for prolonged time, we hypothesize that these might underlie the strong correlation of hyperalgesia to the 2 electrical sinusoidal depolarizing profiles at 7 weeks post rhNGF. Of note, half-sine stimulation induces unsynchronized bursts of APs, which can outlast the actual stimulation period,[27,32] thereby additionally inducing an active receptor potential. Specific mechanisms underlying these bursts include facilitation of high-frequency discharge (>>4 Hz) and generator potentials (involving eg, voltage-sensitive calcium[10] and calcium-dependent chloride channels). The lack of correlation of half-sine-induced pain between 3- and 21-day post rhNGF suggests that instant but rather short-lived changes developing within 3 days contrast structural changes requiring some weeks, although both may contribute to enhanced half-sine wave–induced pain.

Contrasting half-sine wave electrical stimulation of CM nociceptors, CM and CMi nociceptors are both amenable to 4 Hz sine wave stimulation.[12] Unlike CM nociceptors, CMi nociceptors have a particularly low maximum following frequency (<5 Hz) that increased 3 weeks after NGF[39] and which is close to the 4 Hz discharge frequency imposed by our sine wave stimulation. Based on the strong correlation between half-sine wave–induced pain (activation of CM nociceptors) and 4 Hz sinusoidal pain (activation of CM and CMi nociceptors) at day 49, it may be hypothesized indirectly that the rhNGF effect on facilitated CMi-nociceptor activation at 3 week may have declined by 7 weeks such that enhanced sinusoidal pain primarily comprises CM-nociceptor activation at 49 days post rhNGF, but this needs further investigation.

4.3. Adaptation to sustained 4 Hz sinusoidal stimulation

Previous findings in patients with neuropathic pain showed a reduced adaptation or even facilitation of pain to 60-second continuous 4 Hz sine wave stimulation.[12,13] Based on the observation that the "activity-dependent slowing" (ADS) of AP conduction is particularly prominent in CMi nociceptors and strongly reduced following rhNGF injections,[8,22,23] a similarly facilitated response upon CMi-nociceptor activation after intracutaneous rhNGF delivery might be expected. Adaptation to the sustained sine wave stimulation, however, did not change after rhNGF at any assessment day, extending previous findings confined to earlier time points of the model.[31] Apparently, altered nociceptor adaptation to ongoing 4 Hz sinusoidal stimulation in patients depends on neuropathic changes in primary afferents, including characteristic transcriptional changes, spontaneous depolarizing fluctuations, or perhaps altered spike wave forms of the generated APs, all of which contribute to spontaneous pain[18,19] but are insufficiently modeled by a single rhNGF injection.

4.4. Limitations

Our study relies on psycho-physics rather than on direct nerve recordings. The proposed peripheral sensitization by rhNGF is based on previous results showing that hyperalgesia following rhNGF is strictly restricted to the actual injection site[26] and on the stable pain ratings in NaCl-treated control skin reported herein. However, our data cannot exclude that central sensitization might have contributed. Validation studies, perhaps including direct nerve recordings would be required to explicitly demonstrate prolonged changes in the evoked activity of particular C-fiber subclasses.

In summary, the results indicated long-lasting augmented pain responses after single rhNGF injections into human skin particularly upon CM nociceptor stimulation. The likely source for this nociceptor hyperexcitability may be located in the periphery, although altered excitability at CNS level additionally could have contributed. Given the causal role of NGF in localized chronic inflammatory pain, our findings suggest that targeting neuronal sensitization mechanisms could be a particularly promising therapeutic strategy in this condition.

Disclosures

The authors have no conflict of interest to declare.

Acknowledgements

The authors thank Frau Ilona Rossbach for careful proofreading of the manuscript. The study was supported by the German Research Foundation (Deutsche Forschungsgemeinschaft), grant project 397846571 (R.R.) and FOR 2690 (H.J.S., M.S., R.R.) and SFB 1158 (H.J.S., M.S.).

Data availability: Data are available from the corresponding author upon reasonable request.

Supplemental digital content

Supplemental digital content associated with this article can be found online at http://links.lww.com/PR9/A247.

Article history:
Received 28 December 2023
Received in revised form 20 March 2024
Accepted 9 May 2024
Available online 20 September 2024

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