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L.B. Sørensen, MSc[1], P. Gazerani, Ph.D.[2], K.A. Sluka, P.T., Ph.D.[3], T. Graven-Nielsen, Ph.D., DMSc[1*]

[1]Center for Neuroplasticity and Pain (CNAP), Aalborg University, Denmark
[2]Biomedicine, Department of Health Science and Technology, Faculty of Medicine, Aalborg University, Denmark
[3]Neuroscience Institute, The University of Iowa, Iowa City, IA, United States.

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***Corresponding Author:**

Prof. Thomas Graven-Nielsen, PhD, DMSc.

Center for Neuroplasticity and Pain (CNAP)

Department of Health Science and Technology

Faculty of Medicine, Aalborg University

Fredrik Bajers Vej 7 D3, DK-9220 Aalborg, Denmark

Phone: +45 9940 9832, Fax: +45 9815 4008

E-mail: tgn@hst.aau.dk

ABSTRACT

Objective: Nerve growth factor (NGF) is essential for generating and potentiating pain-responses. This double-blinded crossover-study assessed NGF-evoked pain in healthy humans after repeated NGF-injections in tibialis anterior (TA) muscle compared with control-injections of isotonic-saline.

Subjects: Twenty healthy subjects participated in two experimental phases; each consisted of seven sessions over twenty-one days.

Methods: At Day0, Day2, and Day4, a low-dose NGF (1µg) was injected. Daily self-reported muscle pain (Likert scale) was collected. Pressure pain threshold (PPTs), pain evoked by non-ischemic and ischemic muscle-contractions (numerical rating scale, NRS), pressure-pain detection and pain-tolerance thresholds (PDTs, PTTs) to cuff-algometry were recorded before (Day0), 1, 2, 4, 7, 10, and 21 days after the first injection. Temporal summation of pain (TSP) and conditioned pain modulation (CPM) were recorded to assess central pain mechanisms.

Results: Likert-scores remain elevated for 9-days after NGF ($P<0.05$). PPTs at the TA muscle were decreased at Day1 until Day7 after NGF compared with Day0 ($P=0.05$). In subjects presenting with NGF-induced muscle hyperalgesia, pain NRS scores evoked by non-ischemic contractions were higher after NGF at Day4 and Day7 ($P<0.04$) compared with the control condition. At all time-points, higher pain NRS scores were found with ischemic compared with non-ischemic contractions ($P<0.05$). The pain NRS after ischemic contractions was elevated following prolonged NGF-hyperalgesia at Day7 compared with the control condition and Day0 ($P<0.04$). The PDT, PTT, TSP, and CPM remained unchanged during the period of NGF-induced hyperalgesia.

Conclusion: Repeated low-dose NGF injections maintain muscle pain and potentiate pain evoked by ischemic-contractions during prolonged NGF-hyperalgesia.

INTRODUCTION

Nerve growth factor (NGF) is an essential protein that is involved in nociception (1) and clinical pain conditions (2). For example, experimentally induced mechanical hyperalgesia peaks 1-2 days after NGF injections and facilitates pain provoked by the activity of the affected muscle such as daily movement (3,4) or moderate contractions (5,6).

Muscle hyperalgesia has been demonstrated within a few hours after injecting NGF (5µg) in the tibialis anterior (TA) muscle with decreased pressure pain thresholds (PPTs) lasting up to 4-days (5,6). Moreover, prolonged muscle hyperalgesia was induced by three daily NGF injections (3x5µg), with reduced PPTs lasting 6-days (7). Since no dose-response relationship has been established, it is unknown whether repeated injections of low-dose NGF (1µg) to the same site could induce and maintain muscle hyperalgesia.

The initial response to NGF-induced muscle hyperalgesia is likely due to peripheral mechanisms. In contrast, altered central processing is suggested to occur in a later phase, when NGF is present for a longer time, e.g., through retrograde transport of NGF to the cell body (8). NGF promotes the expression of ion channels and receptors such as acid-sensing ion channels (ASICs) and the transient receptor potential vanilloid 1 (TRPV1), which are associated with mechanical allodynia (9) and hyperalgesia (10,11). With the slow retrograde transport (12,13), the estimated duration of such a process would be at least 2-3 days for lower extremity muscles (14).

Recently, it was found that ischemic muscle-contraction yield increased pain one day after five low-dose NGF injections given at baseline (15), suggesting that NGF may influence the responsiveness of chemo-sensitive channels that are active during ischemic-contractions. If more channels were available or further sensitized, ischemic-evoked pain might increase over time with maintained NGF-induced muscle hyperalgesia.

Changes of central pain mechanisms (e.g., pain distribution, descending pain control, or temporal summation of pain) during prolonged NGF-induced muscle hyperalgesia have been partly demonstrated. Pain areas induced by tonic-pressure stimulation expanded progressively with daily NGF injections (5µg) (7) but not with a single low-dose NGF injection (6). Temporal summation of pain (TSP) was facilitated when assessed at the NGF injection-site after one day (4). Interestingly, prolonged NGF-induced hyperalgesia has also been followed by reduced pressure pain sensitivity (5,6). Whether this reflects habituation or a slower normalization of the descending pain control-system remains unknown. Therefore, whether NGF-induced pain excites the descending pain controls systems, is unclear, and a conditioning pain modulation (CPM) paradigm has not been tested with prolonged NGF-hyperalgesia.

This study assessed if low-dose NGF injections into the TA muscle, three times with two days-intervals, would induce prolonged muscle hyperalgesia when controlled by isotonic saline injections. Secondly, if low-dose NGF injections maintain muscle hyperalgesia, it was studied whether such prolonged effect would increase pain evoked by ischemic and non-ischemic muscle-contractions with the involvement of central pain mechanisms. It was hypothesized that prolonged NGF-induced muscle hyperalgesia compared with baseline and control conditions is accompanied by 1) increased muscle pain after ischemic-contractions, 2) enlarged pressure-induced pain areas, 3) facilitated TSP, and 4) impaired CPM.

MATERIALS AND METHODS

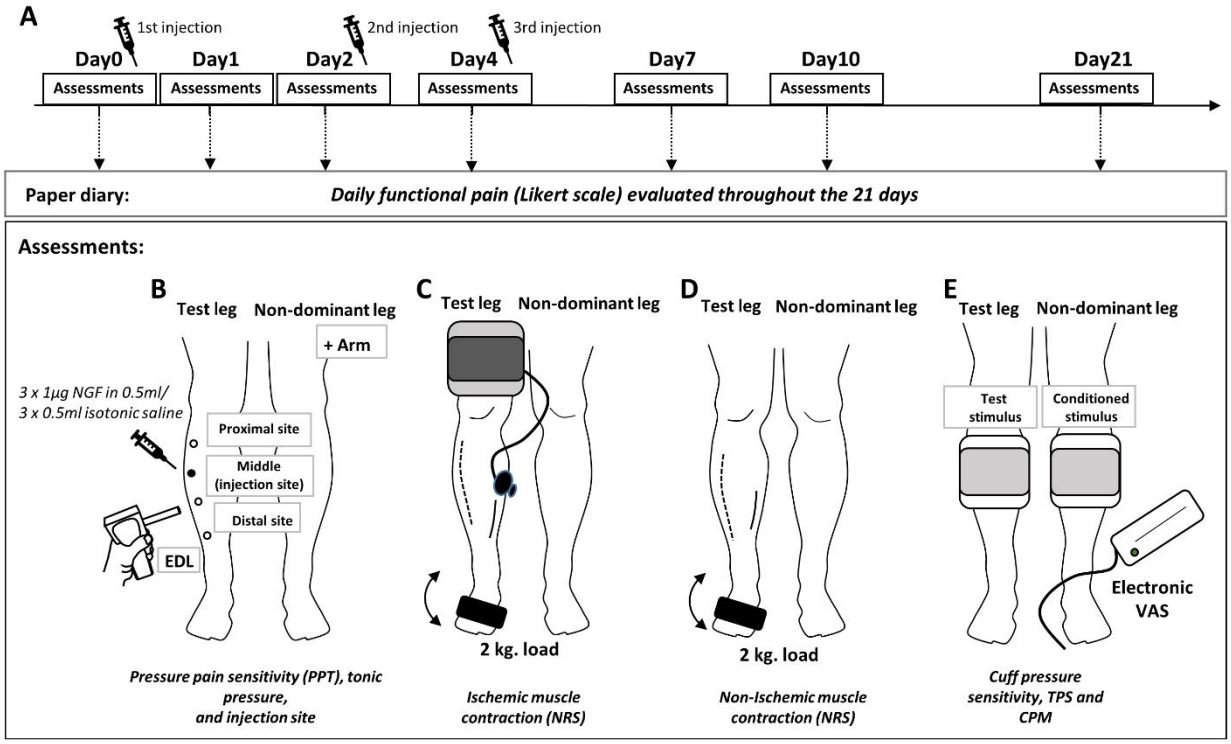
Participants

Twenty healthy participants were included in the study (mean age 26.5±3.0 years, range 20-31 years; eight females). The participants were recruited through social media and advertisements at

Aalborg University in Denmark in the period from February to June 2019. Exclusion criteria included acute or chronic pain, neurologic, musculoskeletal or inflammatory conditions, and muscle soreness at the lower legs assessed by palpation before study participation, or any history of injuries of the legs within the past six months. Participants were advised not to take any non-steroidal anti-inflammatory drugs (NSAIDs), and to avoid strenuous exercise of the legs throughout the study period. All participants received written and verbal information about the study, and all signed a consent form before the first experimental session. The study was performed according to the Helsinki Declaration (16), approved by the North Denmark Region Committee on Health Research Ethics (N-20170007), and was registered at ClinicalTrials.gov (NCT03844243).

Experimental protocol

A crossover, randomized, and placebo-controlled study was designed that included 2 phases, each divided into seven sessions throughout 21-days. A break (i.e., wash-out period) of 4 weeks was



kept between each study phase. All participants received three injections of either NGF (1 µg, 0.5 ml) or isotonic-saline (control, 0.5 ml) into the dominant tibialis anterior (TA) muscle. The first injection was given after baseline assessments on Day0, and the following two injections were injected at the same site after the assessments on Day2 and Day4 (Fig. 1). The injections were randomized between participants in a balanced manner, i.e., 10 received NGF in the first phase and isotonic-saline in the second phase of the study. Before data collection on Day0, participants were introduced to the test procedure and experimental devices.

Figure 1. *Time-line of the seven experimental sessions* ***(A)*** *and the assessment protocol* ***(B, C, D, E)*** *in each phase of the study. Three injections of either NGF or isotonic-saline (control) were given after the assessments at Day0, Day2, and Day4 in the middle TA site. All sessions consisted of the same assessment protocol:* ***(B)*** *Pressure pain thresholds (PPTs) recorded at five assessments sites (proximal, middle, distal, m. extensor digitorum longus (EDL), and m. extensor carpi radialis brevis (arm)), and tonic pressure stimulation assessed at middle TA site (injection site). Pain assessment (numerical rating scale, NRS) of loaded muscle contractions* ***(C)*** *with and* ***(D)*** *without ischemia.* ***(E)*** *Assessment of cuff pressure pain sensitivity, temporal*

summation of pain (TSP), and conditioned pain modulation (CPM) using cuff algometry. NGF: Nerve Growth Factor.

Each experimental session included self-reported muscle pain with daily functional tasks (Likert scale), mechanical pain sensitivity as assessed by pressure algometry and cuff algometry, TSP, and CPM. Additionally, pain drawings after tonic-pressure-induced pain and pain evoked by normal contractions and ischemic contractions were collected. Self-reported pain with daily functional tasks was assessed in the days between assessment sessions by completing a paper diary at home. The same examiner performed the experimental procedure and tests included in the protocol and was blinded to the type of injection. Another examiner prepared and randomized the injections.

Injection protocol

A sterile solution of recombinant human NGF (3 ml vials with 2µg/ml) was prepared by Skanderborg pharmacy, Denmark. NGF was injected into the muscle belly of TA (5,7). In this study, a dose of NGF (1 µg, 0.5 ml) was used. As a control, isotonic-saline (9 mg/ml, 0.5 ml) was injected at the same site in the opposite study phase. The TA muscle and relevant landmarks were identified by manual palpation, and the TA muscle site (middle) was located approximately one-third distal from the lateral femoral epicondyle down to the upper edge of the lateral malleolus (Fig. 1B). The anatomical boundaries of the TA muscle were localized using ultrasound imaging, and the injection site was marked at a 2 cm distance lateral to the tibial bone.

Daily reporting of pain with functional tasks

Participants were asked to complete a paper dairy consisting of a modified 7-point Likert scale for the lower legs to assess muscle pain during the 21 days of each study phase. The scale was defined as: 0, 'A complete absence of pain'; 1, 'A light pain felt only when touched / a vague ache'; 2, 'A moderate pain felt only when touched / a slight persistent pain'; 3, 'A light pain when walking up and down the stairs'; 4, 'A light pain when walking on flat surface'; 5, 'A moderate pain, stiffness or weakness when walking'; 6, 'A severe pain, stiffness or weakness that limits my ability to move' (17).

Hand-held pressure algometry

A hand-held pressure algometer (Somedic, Sösdala, Sweden) with a 1 cm^2 circular rubber tip was used to assess PPTs at five assessment sites. Three sites were chosen over the TA muscle (proximal, middle, and distal), and at the extensor digitorum longus (EDL) muscle on the dominant leg, and the extensor carpi radialis brevis (ECRB) muscle on the non-dominant arm (Fig. 1B). The middle assessment site on the TA muscle was previously defined as the TA muscle injection site. From this site, proximal and distal sites respectively were marked on the same line along with the proximal and distal directions with a distance of 4 cm from the injection site. The site on the EDL muscle was identified lateral to the dominant TA muscle by manual palpation and approximately 20 cm in a straight line proximal from the upper edge of the lateral malleolus. The ECRB muscle was chosen as a proximal and contralateral control site and was identified by palpation. The pressure was applied at a constant rate of 30 kPa/s over each site, and the participants were instructed to press a stop button when the sensation of pressure first becomes painful. Each site was assessed three times, with approximately 30 s interval between each stimulus, and the

average of three readings per site was used for statistical analysis. Based on recent low-dose NGF studies (6,15), muscle hyperalgesia was defined as a reduction in PPTs of ≥ 27% from baseline to Day1, and this was used for further NGF- responder analysis.

Tonic pressure-induced pain

A 30-s tonic pressure stimulation at 120% of the PPTs recorded in each session was used to evaluate the effect of a supra-threshold pain stimulation (18); this was applied at the TA assessment site (injection site) using the hand-held pressure algometer (Somedic, Sösdala, Sweden). The 120% stimulation intensity was reached within a few seconds of stimulation before this was held constant for 30 seconds. To evaluate the extension of pressure pain areas, participants filled in a digital body chart (NavigatePain, Denmark), and drew areas of pain during the tonic stimulation. The size of the pain area (pixels) was used for analysis.

Non-ischemic and ischemic contraction evoked pain

Participants performed a repeated sequence of muscle contractions with their dominant leg while lying in a supine position on a bed. Participants were instructed to perform 20 static TA muscle contractions within 2 min. A metronome controlled the speed of the task (60 beats per minute), and the participants were encouraged to hold the foot in a fully flexed position for 2 s before relaxing the foot for 4 s. A load of 2 kg was strapped to the distal part of the foot to resist dorsiflexion (5). Upon completion of the task, the participants verbally rated their pain intensity on a numerical rating scale (NRS), with the anchors of 0 for ‘no pain’ and 10 for ‘worst pain imaginable.’ After a small break, ischemia was induced in the same leg by application of a manual occlusion cuff (VBN Medizintechnik GmbH, Germany, cuff size: 107 cm) as described previously

(19). Briefly, the cuff was mounted proximal to the knee and inflated to 250 mmHg. Within the first 2 min, the participants were instructed to perform the same sequence of 20 static TA muscle contractions (2 s hold, 4 s relax) and rate their pain intensity on the NRS. The NRS scores following non-ischemic and ischemic muscle contractions were used for analysis.

Cuff pressure pain sensitivity

A computer-controlled cuff algometer (Nocitech, Aalborg University, Denmark), consisting of two 13 cm wide pressure cuffs (VBM Medizintechnik GmbH, Sulz am Neckar, Germany), and an electronic visual analogue scale (eVAS, Aalborg University, Denmark) anchored at 0 cm 'no pain' and 10 cm 'worst pain imaginable', were used to assess cuff pressure pain sensitivity, TSP, and CPM (Fig. 1E). The cuffs were placed over each lower leg with the upper edge of the cuff covering the level of the proximal assessment site on the TA muscle. The cuff pressure was increased by one kPa/s, with maximum pressure at 100 kPa. Participants were instructed to continuously rate their pain on the eVAS when the sensation of pressure changed into the first pain and then press a stop button when they could not tolerate the pain any longer. The pressure pain detection threshold (PDT) was defined as the pressure at which the eVAS score exceeded 1 cm (20), and the pain tolerance threshold (PTT) was defined as the pressure when participants pressed the stop button. If PTT was not reached, the maximum pressure of 100 kPa was used for further analysis. PDT and PPT were assessed bilaterally.

Temporal summation of pain

A series of 10 pressure stimulations (1s duration, 1s intervals) were applied to the participant's dominant leg using the pressure cuff algometer to assess TSP. Each stimulus was applied at the

level of PTT recorded in the same session. The participants were instructed to continuously rate their pain intensity on the eVAS during the sequential stimulations without returning to zero between cuff inflations. The eVAS scores were normalized by subtraction of the 1st VAS score in the series, and then mean VAS from the 2-4 score (VAS-I), and the 8-10 score (VAS-II) were calculated. The TSP-effect was defined as the difference between VAS-I and VAS-II (i.e., VAS-II minus VAS-I) (21), and used for further analysis.

Conditioned pain modulation

CPM was assessed by changes in PDT on the dominant leg (also receiving injections) with a conditioning stimulation applied to the contralateral leg. The conditioning stimulus was induced by inflation of a pressure cuff that maintained pressure at 70% of PTT during the test (of that day). Few seconds after the inflation of the conditioning stimulation cuff, the cuff on the dominant leg was inflated with a rate of 1 kPa/s, and PDT and PTT were reassessed, respectively. Participants were instructed to focus on their dominant leg and rate their pain using the eVAS. Both cuffs deflated when the participant pressed the stop button or when the cuff system reached the 100 kPa limit. The CPM-effect was calculated as the difference between PDTs with and without the conditioning stimulus.

Statistics

Data are presented as mean and standard deviation (SD) in text and mean and standard error of the mean (SEM) in figures and tables. All statistical analyses were completed in SPSS (IBM SPSS

version 25), and the significance level was set to P≤0.05. Data were controlled for normality using the Shapiro-Wilk test and analyzed with parametric or nonparametric tests accordingly.

Daily reporting of pain (Likert score) was analyzed across time using Friedman test of variance, followed by Wilcoxon signed-rank tests and Bonferroni correction. PPTs recorded from the TA muscle were analyzed by a 3-way repeated-measure analysis of variance (RM-ANOVA) where the within-subject factors were: *condition* (NGF vs. saline), *site* (proximal, middle, distal), and *time* (sessions). A 2-way RM-ANOVA with factors *condition* and *time* was used to analyze PPT values from the EDL and ECRB muscles, respectively. PPTs across sessions of the TA muscle were analyzed by 2-way ANOVA with factors *condition* and *site*, and PPTs across sessions of the EDL and ECBR muscles were each analyzed using a paired *t*-test.

The pain NRS scores following non-ischemic and ischemic muscle contractions were analyzed by 3-way RM-ANOVA with the within-subject factors: *condition* (NGF vs. saline), *ischemia* (with vs. without), and *time* (sessions).

PDTs and PTTs assessed by cuff algometry on the test leg and non-dominant leg, TSP-effect, and CPM-effect were analyzed by 2-way RM-ANOVAs with the factors: *condition* (NGF vs. saline), and *time* (sessions). If the sphericity assumption was violated, the Greenhouse-Geiser correction was used. Post-hoc analysis for multiple comparisons with Bonferroni was used for all ANOVAs when significant factors or interactions allow.

As reported in a recent NGF crossover study (15), a 2-step confirmatory analysis was performed to check that the order of injection type (i.e., whether the NGF injections were given in the first phase or the second phase) did not affect the outcomes in the opposite phase of the study. An independent *t*-test comparing the sum of PPTs, Likert scores, PDTs, PTTs, NRS, and pain

areas across sessions in both phases between the group that received NGF first and then saline (NGF + Saline) and the group that first received saline and then NGF (Saline + NGF) was performed.

RESULTS

Three participants did not finalize both phases of the study and are not included in the analysis. Besides this, no protocol deviations have been noted.

Daily reports of pain

Increased Likert scores of pain during daily activities were reported in the leg injected with NGF from Day3 to Day9 when compared with pre-injection at Day0 (Fig. 2, Friedman: $X^2(21)=161.88$, $P<0.01$), and different from control injection of isotonic-saline at Day3, 6, and 7 (Wilcoxon, Post-hoc: $P\leq0.05$). In general, overall low Likert pain scores were observed after the low-dose repeated NGF injections, and 6 out of 17 participants reported almost no activity evoked pain (Likert score ≤1) throughout the 21 days.

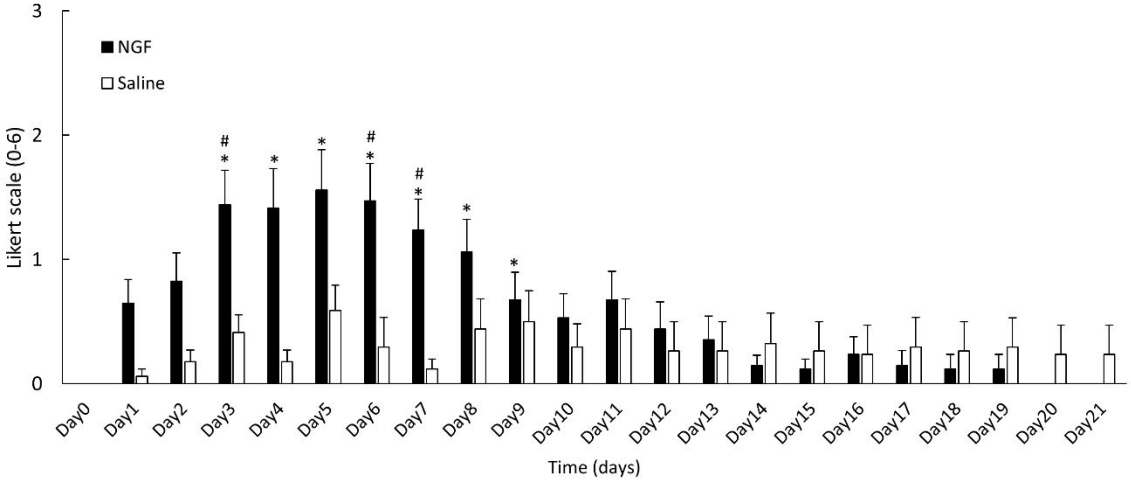


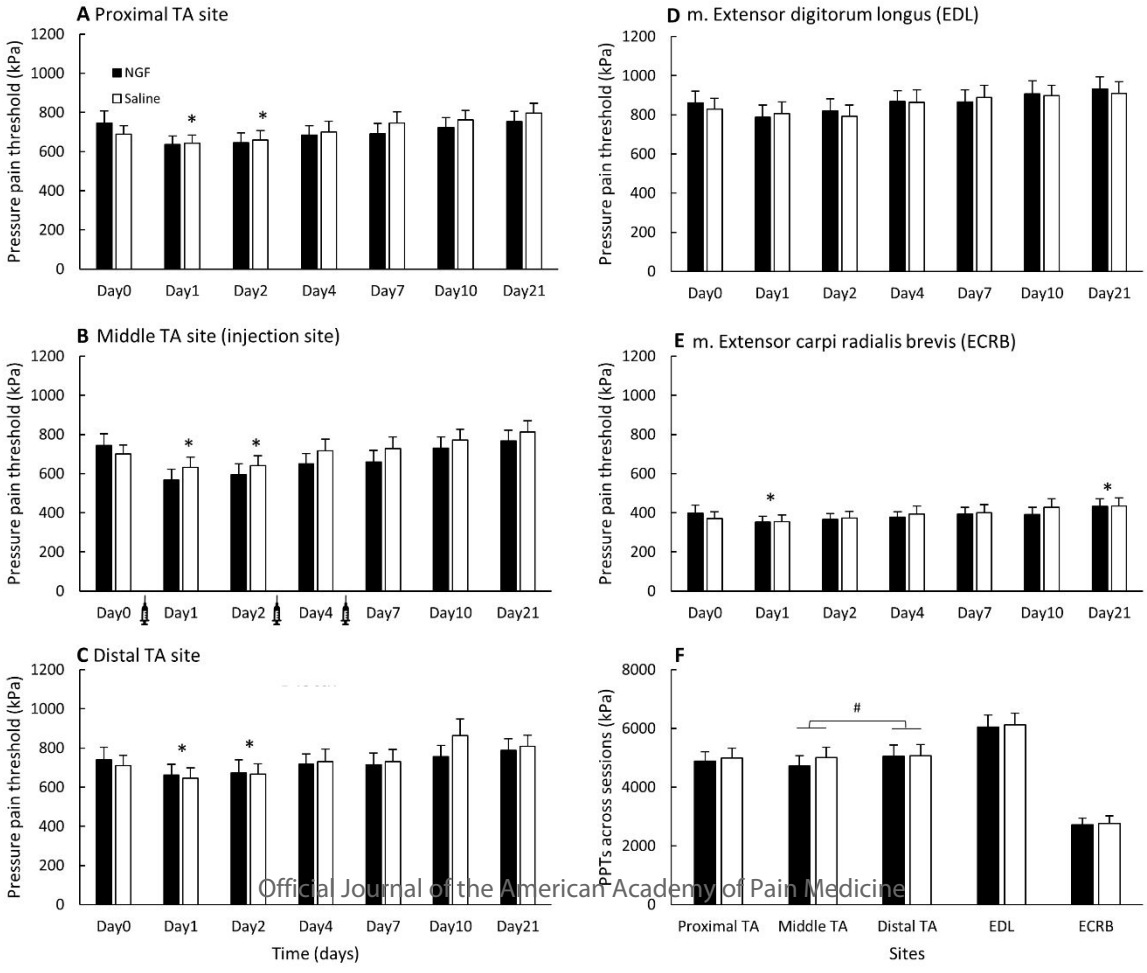
Figure 2. *Mean (+SEM, n=17) Likert Scale scores of the pain diary for the leg injected with NGF (black bars) and isotonic-saline (control condition, open bars). Significantly different compared with pre-injection (Day0; *, P≤0.05), or compared with saline (#, P≤0.05). NGF: Nerve Growth Factor*

Pressure pain sensitivity

The ANOVA of PPTs at the TA muscle showed a main effect of time with decreased PPTs at Day1 and Day2 compared with PPTs at baseline (Day0), before injections (Fig. 3A, B, C, ANOVA: F=1.12, P=0.01).

The ANOVA of PPTs for the EDL muscle showed no significant interaction between condition and time (Fig. 3D, ANOVA: F0.61, P=0.50), nor was there any main effect for condition (P=0.77) or time (P=0.07). For the ECRB muscle, changes in PPTs were seen at Day1 and Day21 when compared with PPTs at Day0 (Fig. 3D, ANOVA: F=1.20, P=0.05, P=0.02).

The ANOVA of PPTs across sessions showed an interaction between condition*site for the TA muscle (Fig. 3F, ANOVA: F=3.83, P=0.03), demonstrating that the PPTs at the middle site (injection



site) were lower (i.e., more decreased) than the PPTs at the distal sites for both conditions. There was no significant difference between NGF and the control injections of isotonic-saline at any of the three TA sites (P>0.05). Similarly, there was no difference in PPTs across sessions between the leg injected with NGF and the control leg for the EDL (t(16) = −0.29, P= 0.77) or ECRB (t(16) = −0.28, P=0.78) muscles.

Figure 3. *Mean (+SEM, n=17) pressure pain thresholds (PPTs) following the injections of NGF (black bars) and isotonic-saline (open bars) at assessments sites over the TA muscle:* ***(A)*** *proximal site,* ***(B)*** *middle site (injection site),* ***(C)*** *distal site, and adjacent muscle:* ***(D)*** *m. extensor digitorum longus (EDL), and control site* ***(E)****: m. extensor carpi radialis brevis (ECRB). PPTs were recorded at baseline (Day0) before injections and on Day1, 2, 4, 7, 10, and 21 days after injections.* ***(F)*** *Illustrates PPTs across sessions. The syringes indicate the time of injections at Day0, 2, and 4. Significantly different compared to Day0 (*, P<0.05). Significant difference between middle TA site and distal TA site (#, P=0.03). NGF: Nerve Growth Factor, kPa: kilopascal.*

NGF-responder analysis

Four of the 6 participants (2 females) reporting a Likert Pain score ≤1 also showed less pronounced muscle hyperalgesia after the injections of NGF. All four participants showed increased PPTs to either 1 of the injections or all 3 NGF injections. Based on the combination of a low Likert pain score, increased PPTs and/or a deficient decrease in PPTs (<27%) at Day1, four participants (out of 17) were defined as not responding to the low-dose NGF and do not qualify for the remaining hypotheses of the study (requiring a condition of hyperalgesia). Thus, for the subsequent analysis, 13 participants with deep-tissue hyperalgesia are included.

PPTs from the 13 responders (mean age 26.9±3.1 years, range 23-31 years; five females) showed prolonged muscle hyperalgesia following NGF injections from Day1 until Day7 over the TA muscle compared with Day0, pre-injection (P<0.05, data are presented in supplementary material,

Fig. S1). Pain areas following 30s tonic pressure stimulations were not significantly different across condition or time (results are shown in supplementary material, Fig. S2).

Non-ischemic and ischemic contraction evoked pain

The ANOVA for the non-ischemic and ischemic contraction evoked NRS pain scores showed a 3-way interaction between condition*ischemia*time (Fig. 4, ANOVA: F=3.33, P=0.01). For the non-ischemic contractions, higher NRS pain scores were reported at Day4 (Post-hoc: P=0.04), and Day7 (Post-hoc: P=0.02) in the NGF sensitized leg when compared with the control injection. At all-time points, higher NRS pain scores were reported when muscle contractions were performed with ischemia for both the leg injected with NGF (post-hoc: P<0.05), and control injection (post-hoc: P<0.05) when compared with muscle contractions performed without ischemia. Interestingly, after ischemic contractions on Day7, a higher NRS pain score was reported in the leg injected with NGF, when compared with both pre-NGF injection at Day0 (post-hoc: P=0.04) and with control injection the same day (post-hoc: P=0.01).

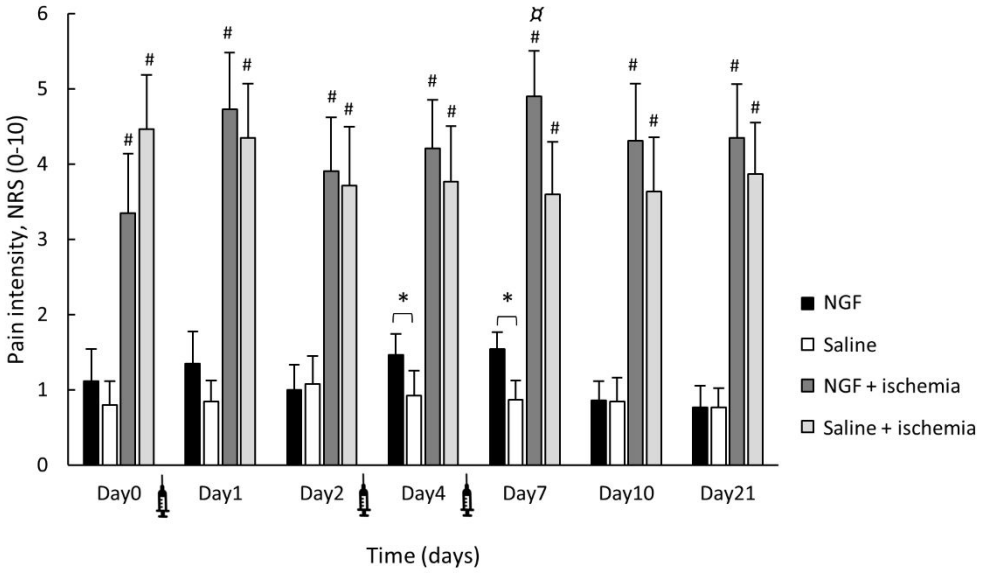


Figure 4. *Mean (+SEM, n=13) of the Numerical Rating Scale (NRS) pain score following non-ischemic contractions when the leg was hyperalgesic by NGF (black bars) and after control injection of isotonic-saline (open bars), following ischemic muscle contractions performed when the leg was hyperalgesic by NGF (dark grey bars), and after the injections of isotonic saline (light grey bars). The syringes indicate the time of injections. Significantly higher compared to non-ischemic muscle contractions (#, P<0.05). Significantly increased compared with isotonic saline following non-ischemic contractions at Day4 and Day7 (*, P<0.04). Significantly increased compared with isotonic-saline following ischemic muscle contractions at Day7 and Day0 (¤, P<0.04). NGF: Nerve Growth Factor*

Cuff pressure pain sensitivity

The ANOVA results of PDTs and PTTs for the test leg showed no significant interaction between condition and time (Fig. 5A, PDT, ANOVA: F=0.79, P=0.58; Fig. 5B, PTT, ANOVA: F=0.41, P=0.73), or any main effect of either condition (P=0.99 and P=0.76) or changes over time (P=0.42 and P=0.40).

The ANOVA results of the PDTs and PTTs for the non-dominant leg (not injected) also showed no significant interaction between condition and time (Fig. 5C, PDT; ANOVA: F=0.67, P=0.67, PPT; Fig. 5D, ANOVA: F=0.62, P=0.71), or any main effects of condition (P=0.40 and P=0.56) or time (P=0.46 and P=0.43).

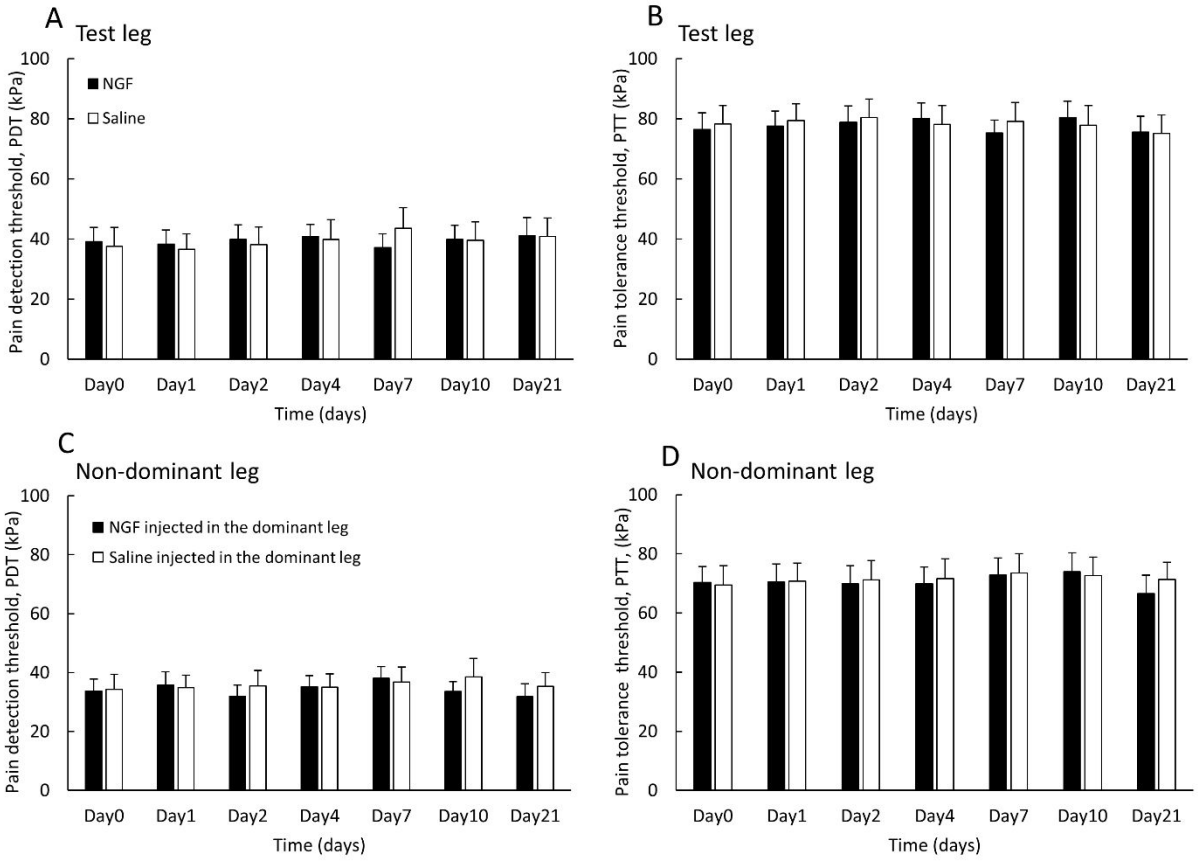


Figure 5. *Mean (+SEM, n=13) pain detection thresholds (PDTs, **A**) and pain tolerance thresholds (PTTs, **B**) for the test leg injected with NGF (black bars) and isotonic-saline (white bars), and similar for the PDT at the non-dominant leg (C), and PTT at the non-dominant leg (**D**). NGF: Nerve Growth Factor, kPa: kilopascal.*

Temporal summation of pain

The ANOVA of the TSP-effect showed no significant interaction between condition and time (Table 1, ANOVA: F=1.39, P=0.26), or any main effect of either condition (P=0.89) or changes over time (P=0.70).

Conditioned pain modulation

The ANOVA of the CPM-effect assessed as the difference between PDT with minus without the conditioning stimulus showed no interaction between condition and time (Table 1, ANOVA: F=0.83, P=0.53). Furthermore, there was no change over time (P=0.17), or effect of condition

(P=0.85). PDTs and PPTs with and without the conditioning stimulus are shown in Fig. S3 in supplementary materials.

Experimental session	NGF TPS-effect (cm)	Saline TPS-effect (cm)	NGF CPM effect (kPa)	Saline CPM effect (kPa)
Day0	*0.63±0.36*	0.83±0.28	6.81±4.25	*4.55±1.48*
Day1	*0.64±0.23*	0.41±0.14	8.65±3.54	*12.08±2.97*
Day2	*0.68±0.27*	0.47±0.23	8.43±2.26	*8.45±2.58*
Day4	*0.62±0.27*	0.49±0.17	10.06±4.39	*8.24±3.88*
Day7	*0.42±0.21*	0.66±0.25	8.94±2.67	*15.61±2.99*
Day10	*0.44±0.22*	0.64±0.19	15.61±2.97	*8.60±4.92*
Day21	*0.48±0.20*	0.52±0.19	4.47±1.63	*2.88±3.26*

Table 1. *Mean (±SEM, n=13) TSP-effect (VAS-II minus VAS-I) and CPM-effect (PDT with minus without conditioning) assessed at baseline (Day0) and the following experimental sessions after the injections of NGF or control injection of isotonic-saline. NGF: Nerve Growth Factor, kPa: kilopascal.*

Carry-over effect

There was no significant difference in any of the outcome measures of PPTs (proximal, middle, and distal sites), Likert score, PDT, PTT, NRS, and local pain areas to pressure stimulation when comparing the sum across both phases (phase 1 + phase 2) between the participants who received NGF in the first phase (Group: NGF + Saline, n=6), and the participants who received NGF in the second phase of the study (Group: Saline + NGF, n=7). Results are shown in supplementary material, Table S1.

DISCUSSION

This study showed that low-dose NGF injections in healthy humans maintain muscle pain and induce less pronounced muscle hyperalgesia. However, four out of 17 participants did not respond to the low-dose NGF. Moreover, in participants responding with muscle hyperalgesia, pain evoked by non-ischemic and ischemic muscle contractions was higher during NGF-sensitization. Further, the ischemic muscle contractions evoked pain that was increased after prolonged NGF-sensitization. Cuff pressure pain sensitivity was not different between NGF and control condition, and the prolonged period of NGF-sensitization did not significantly alter temporal summation of pain and conditioning pain modulation.

Muscle pain with daily function

In the present study, higher self-perceived muscle pain evoked with daily activities of the lower legs was reported in the phase when NGF was injected, which is consistent with previous NGF studies (5,14). Moreover, the repeated injections of low-dose NGF showed a prolonged period with muscle pain lasting up until Day9, which has similarly been demonstrated by Hayashi et al. (7) using the higher dose (5µg) injected on three consecutive days. However, pain intensity is less pronounced in the present study. Nonetheless, six out of 17 participants reported an extremely week activity-evoked pain after the low-dose NGF injections. It remains speculative whether these participants simply did not respond to the lower NGF dose, or whether a certain expectation of

NGF influenced them. However, the order of injection type did not affect the pain response in this study.

NGF-induced muscle hyperalgesia

The repeated injections of low-dose NGF caused mild muscle hyperalgesia in the TA muscle that was present until Day2 after the first NGF injection and, additionally, not significantly different from control injections. The sum of PPTs across sessions also showed higher muscle pain sensitivity (i.e., more decreased PPTs) at the middle injection site for both conditions compared with the distal TA site. Changes in PPTs after control injections of isotonic saline have also been observed in prior studies 1-2 days post-injection. However, the decrease in PPTs after isotonic saline was smaller compared with the reduction of PPTs after NGF injection (15,22). As the same phenomenon was observed in a non-injected muscle, it was also suggested to reflect a possible physiological adaptation to repeated measures (22). A recent low-dose NGF study (15) indicated that a potential release of NGF during strenuous muscle contraction would account for possible muscle hyperalgesia in the control condition. However, since ischemic and non-ischemic contractions were performed in every session, this would not explain why only Day1 and Day2 were affected in the current study.

Interestingly, four participants did not respond to the lower dose of NGF with muscle hyperalgesia but instead showed reduced sensitivity (i.e., PPTs seemed to increase) after the injections. In previous studies, the duration of NGF-induced muscle hyperalgesia has been suggested to be dose-dependent and different between larger and smaller muscles (5,22). However, in two recent NGF studies (6,15), the low-dose NGF injections (1µg) induced similar muscle hyperalgesia in the TA muscle, as previously demonstrated by Andersen et al. (5) injecting

5µg into the TA muscle. As the majority of the participants included in the current study developed muscle hyperalgesia and pain, it is unknown why those four participants responded to the NGF injections differently.

In the 13 participants responders, the repeated NGF injections induced muscle hyperalgesia after one day that maintained until Day7 at all sites of the TA muscle with a more pronounced decrease in PPTs at the site of injection (middle site) at Day1 compared with the distal site. Compared with a previous NGF study, spatially distributing NGF at one time-point (6), the present study illustrates that repeated injections of low-dose NGF were able to prolong muscle hyperalgesia in those participants that responded to the lower dose NGF. However, the degree of muscle hyperalgesia did not change with the number of injections. When higher levels of NGF are available at the site of injection, no findings indicate that a further reduction in mechanical hyperalgesia would occur. Instead, more NGF (e.g., upregulation of NGF) has been found critical for maintaining muscular hyperalgesia (23). Consistent with previous NGF studies, repeated injections of the higher-dose NGF (5µg) showed prolonged muscle hyperalgesia (7,24). In these latter studies (7,24), it was suggested that the prolonged duration, most likely was due to the reapplication of NGF. However, whether repeated application alone would support the process of retrograde transport, and through this, sustain NGF-induced muscle hypersensitivity, is still unclear.

Although NGF was injected in the middle of the TA muscle, the proximal and distal sites also were affected, albeit this was less pronounced compared with the effects at the injection site. A widespread effect of NGF, based on a central mechanism, has further been suggested to underlie such findings (5). On the contrary, there was no extension of the local pain areas with prolonged NGF-induced hyperalgesia after the tonic pressure stimulation in the current study. A recent low-

dose NGF study (6) also showed no extension of local pain areas after tonic pressure stimulation on the TA muscle. In contrast, Hayashi et al. (7) showed larger pressure pain areas over time with repeated NGF (5µg) injections. However, such changes could result from the high-dose NGF injections or the relatively higher pressure stimulation given post-NGF injection as compared with the lower pressure intensity given in the current study (relative to the pain sensitivity of the day).

For the EDL muscle pain sensitivity, no significant changes were found in the present study, which likewise has been demonstrated in a prior low-dose NGF study (6). Although changes at the ECRB muscle were found on Day1 and Day21 in this study, it is unlikely that these stem from the injection of NGF, as previous studies confirm that the sites located extra-segmentally are not affected by NGF (25).

Contraction-evoked pain responses

As previously demonstrated, pain evoked by normal contractions of the TA muscle has been reported after NGF-induced muscle hyperalgesia (3,5,26), which is unique for the NGF model and not observed with other injection-based pain models (27). In prior studies, the evoked pain was present 3 hours after NGF injection and lasted up until Day7 during the same time when muscle hyperalgesia was present. In the present study, normal contractions of the TA muscle evoked pain in the NGF injected leg that was significantly higher at Day4 and Day7 in the thirteen participants who responded with muscle hyperalgesia.

Moreover, in a recent study (15), contraction-evoked pain further increased 1-day post-NGF injection compared with both ischemic muscle contractions performed at baseline (pre-injection) and with a non-sensitized muscle (i.e., control muscle injected with isotonic-saline). Therefore, in the current study, it was speculated whether evoked ischemic-contraction induced pain would be

further facilitated over time due to prolonged NGF-sensitization. At all time-points, higher pain was reported with ischemic muscle contractions for both the NGF injected leg and control leg in this study, when compared with contractions performed without ischemia. Only on Day7, the ischemic contraction-evoked pain was further increased with the NGF injected leg compared with the control muscle. In a prior NGF study (15), it was suggested that the performance of ischemic muscle contractions, would include the activation of chemo-sensitive channels, such as TRPV1 and ASICs present on the sensitized muscle nociceptors, increasing the evoked ischemic contraction-induced pain response. Hence, such an early response could be speculated to result from peripheral mechanisms.

In contrast, significantly more ischemic contraction-induced pain was reported later on Day7 with maintained NGF-sensitization in this study. Although that more NGF would be available for retrograde transport with repeated injections, it can only be speculated whether this would account for this Day7 response. However, a possible change over days would be captured within this time of testing in the current study if such a process would occur after 2-3 days.

Cuff pressure pain sensitivity during prolonged NGF-sensitization

No significant changes over time were found for either PDTs or PTTs, as values remained almost the same across sessions for both conditions. Interestingly, reduced sensitivity to hand-held pressure stimulation, 14-21 days after NGF injection, has been assessed in prior studies (5,6), suggesting this to result from the repeated testing or familiarization of the test procedure. Therefore, increased PDTs and PTTs could have been expected in the current study as cuff pain thresholds generally adapt to the repetitive pressure stimuli over time (28). Moreover, pain threshold values recorded by the hand-held algometer are recorded directly over the affected area (28). In contrast, cuff algometry stimulates a larger volume of the leg, and a higher proportion

of deep-tissue afferents are assessed. Hence, cuff algometry and hand-held pressure stimulation are two different methods for determining pressure pain sensitivity.

Across time or condition, no significant difference was found in the CPM-effect, suggesting that the inhibitory pain systems were not affected by prolonged NGF-induced sensitization in this study. The muscle pain induced by NGF is comparable to the pain observed after delayed onset muscle soreness (DOMS), and further pain is only evoked during movement. A recent study using a DOMS pain model showed no influence on the CPM-effect, suggesting that the DOMS pain intensity might have been insufficient to alter the CPM significantly (29). Similarly, the low muscle pain intensity (Likert Pain score) reported after the low-dose NGF injections, could also be the reason why CPM was not affected by NGF in this study. In contrast, moderate pain intensity evoked by prolonged noxious stimulation (e.g., capsaicin) in healthy participants was recently shown to reduce CPM-effect significantly (30).

Temporal summation of pain

There was no significant effect on temporal summation to repetitive pressure pain stimulations during prolonged NGF-sensitization in the current study. This is in contrast to prior NGF studies, in which TSP has facilitated 1-day after repeated NGF injections (7), and following a pain model combining NGF-sensitization with DOMS (4). Importantly, different methodologies were used in the prior studies to induce TSP by a computer-controlled pressure algometer, causing pressure locally at injections site with the same pressure intensity as the PPTs assessed at baseline. On the contrary, each stimulation in the present study was applied at a more extensive application area with the pressure intensity adjusted to the PPTs recorded in the particular session.

Conclusion

Intramuscular administration of low-dose NGF injections every second day (for four days) was able to maintain low-intensity muscle pain but did not sufficiently induce muscle hyperalgesia in all participants included in this present study or showed significant changes of central pain mechanisms with prolonged low-dose NGF application. However, in participants responding with hyperalgesia to the low-dose NGF, pain evoked by ischemic muscle contractions was further facilitated during the prolonged period of NGF-sensitization. Whether muscle hyperalgesia is maintained locally by the reapplication of NGF or sustained by a central component related to retrograde transportation of NGF remains unknown. More research on healthy subjects and translational studies are needed to clarify the mechanisms behind the long-lasting effect of NGF and the facilitative response on ischemic pain to fully acknowledge NGF as a vital substance with the potential clinical implication in conditions such as chronic inflammation or ischemic pain.

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Conflict of Interest and Disclosure

Aalborg University partly owns Nocitech. Otherwise, no conflicts are to declare.

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Tables

Table 1. Mean (±SEM, n=13) TSP-effect (VAS-II minus VAS-I) and CPM-effect (PDT with minus without conditioning) assessed at baseline (Day0) and the following experimental sessions after the injections of NGF or control injection of isotonic-saline. NGF: Nerve Growth Factor, kPa: kilopascal.

Figure legends

Figure 1. Time-line of the seven experimental sessions **(A)** and the assessment protocol **(B, C, D, E)** in each phase of the study. Three injections of either NGF or isotonic-saline (control) were given after the assessments at Day0, Day2, and Day4 in the middle TA site. All sessions consisted of the same assessment protocol: **(B)** Pressure pain thresholds (PPTs) recorded at five assessments sites (proximal, middle, distal, m. extensor digitorum longus (EDL), and m. extensor carpi radialis brevis (arm)), and tonic pressure stimulation assessed at middle TA site (injection site). Pain assessment (numerical rating scale, NRS) of loaded muscle contractions **(C)** with and **(D)** without ischemia. **(E)** Assessment of cuff pressure pain sensitivity, temporal summation of pain (TSP), and conditioned pain modulation (CPM) using cuff algometry. NGF: Nerve Growth Factor.

Figure 2. Mean (+SEM, n=17) Likert Scale scores of the pain diary for the leg injected with NGF (black bars) and isotonic-saline (control condition, open bars). Significantly different compared with pre-injection (Day0; *, P≤0.05), or compared with saline (#, P≤0.05). NGF: Nerve Growth Factor

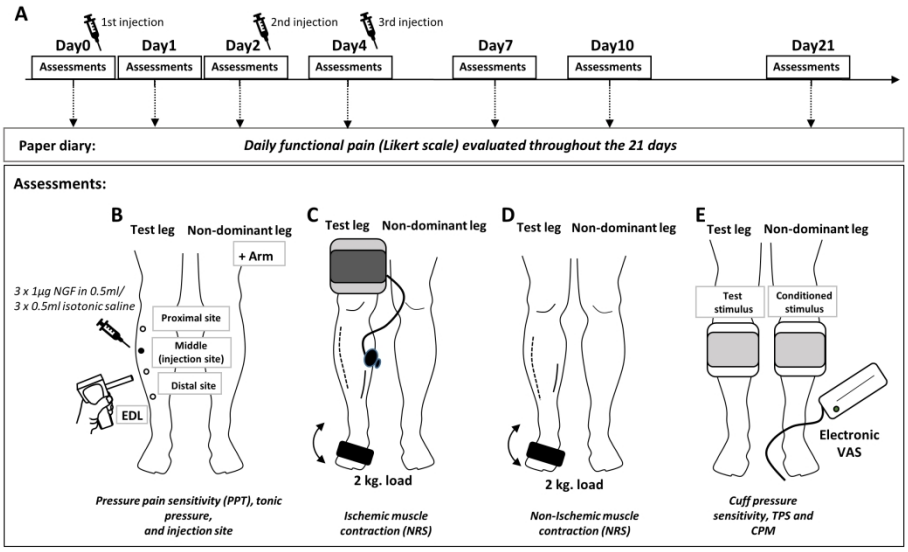
Figure 3. Mean (+SEM, n=17) pressure pain thresholds (PPTs) following the injections of NGF (black bars) and isotonic-saline (open bars) at assessments sites over the TA muscle: **(A)** proximal site, **(B)** middle site (injection site), **(C)** distal site, and adjacent muscle: **(D)** m. extensor digitorum longus (EDL), and control site **(E)**: m. extensor carpi radialis brevis (ECRB). PPTs were recorded at baseline (Day0), before injections, and

on Day1, 2, 4, 7, 10, and 21 days after injections. **(F)** Illustrates PPTs across sessions. The syringes indicate the time of injections at Day0, 2, and 4. Significantly different compared to Day0 (*, P<0.05). Significant difference between middle TA site and distal TA site (#, P=0.03). NGF: Nerve Growth Factor, kPa: kilopascal.

Figure 4. Mean (+SEM, n=13) of the Numerical Rating Scale (NRS) pain score following non-ischemic contractions when the leg was hyperalgesic by NGF (black bars) and after control injection of isotonic-saline (open bars), following ischemic muscle contractions performed when the leg was hyperalgesic by NGF (dark grey bars), and after the injections of isotonic saline (light grey bars). The syringes indicate the time of injections. Significantly higher compared to non-ischemic muscle contractions (#, P<0.05). Significantly increased compared with isotonic saline following non-ischemic contractions at Day4 and Day7 (*, P<0.04). Significantly increased compared with isotonic-saline following ischemic muscle contractions at Day7 and Day0 (¤, P<0.04). NGF: Nerve Growth Factor

Figure 5. Mean (+SEM, n=13) pain detection thresholds (PDTs**, A**) and pain tolerance thresholds (PTTs, **B**) for the test leg injected with NGF (black bars) and isotonic-saline (white bars), and similar for the PDT at the non-dominant leg (C), and PTT at the non-dominant leg (**D)**. NGF: Nerve Growth Factor, kPa: kilopascal.

Experimental session	NGF TPS-effect (cm)	Saline TPS-effect (cm)	NGF CPM effect (kPa)	Saline CPM effect (kPa)
Day0	*0.63±0.36*	0.83±0.28	6.81±4.25	*4.55±1.48*
Day1	*0.64±0.23*	0.41±0.14	8.65±3.54	*12.08±2.97*
Day2	*0.68±0.27*	0.47±0.23	8.43±2.26	*8.45±2.58*
Day4	*0.62±0.27*	0.49±0.17	10.06±4.39	*8.24±3.88*
Day7	*0.42±0.21*	0.66±0.25	8.94±2.67	*15.61±2.99*
Day10	*0.44±0.22*	0.64±0.19	15.61±2.97	*8.60±4.92*
Day21	*0.48±0.20*	0.52±0.19	4.47±1.63	*2.88±3.26*



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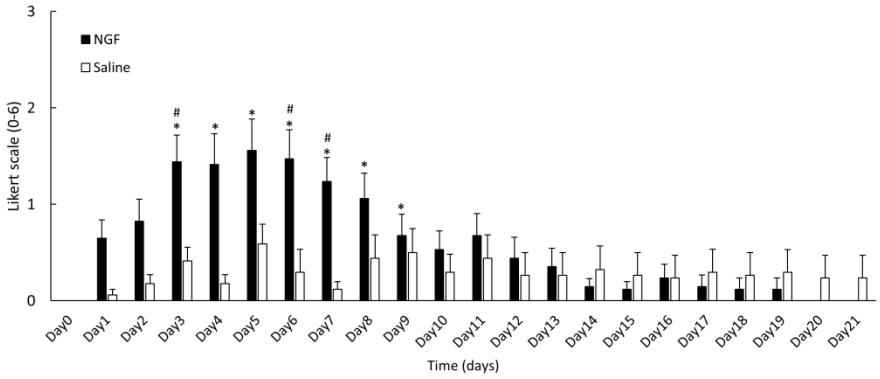

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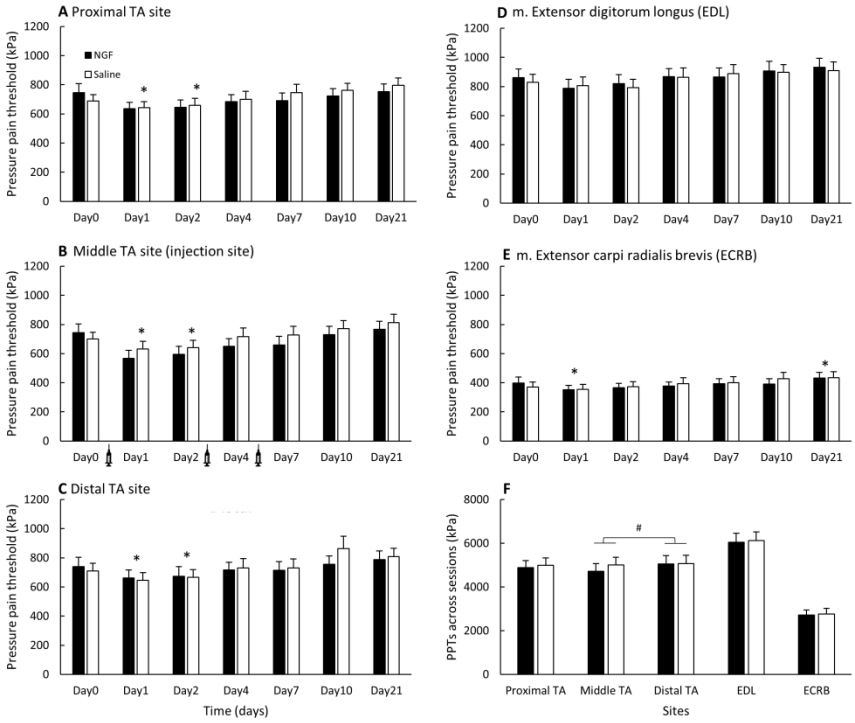

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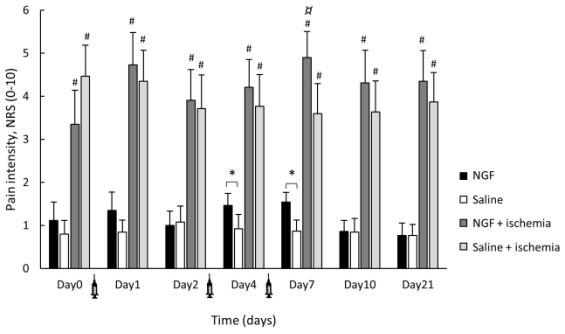

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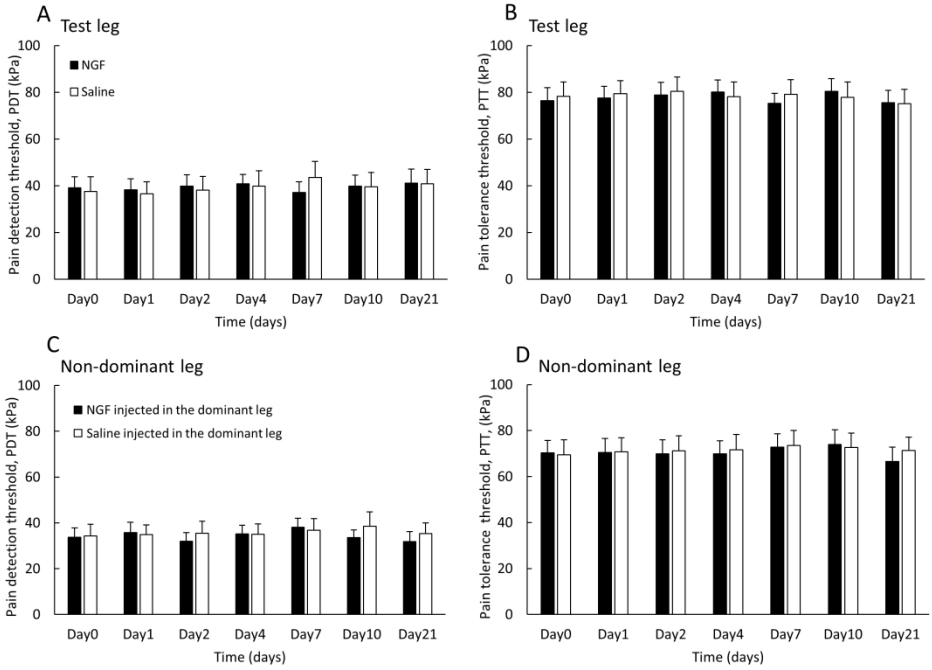

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Supplementary material

REPEATED INJECTIONS OF LOW-DOSE NERVE GROWTH FACTOR (NGF) IN HEALTHY HUMANS MAINTAIN MUSCLE PAIN AND FACILITATE ISCHEMIC-CONTRACTION EVOKED PAIN

L.B. Sørensen, MSc[1], P. Gazerani, PhD [2], K.A. Sluka, P.T., Ph.D[3], T. Graven-Nielsen, PhD, DMSc[1*]

[1]Center for Neuroplasticity and Pain (CNAP), Aalborg University, Denmark

[2]Biomedicine, Department of Health Science and Technology, Faculty of Medicine, Aalborg University, Denmark

[3]Neuroscience Institute, The University of Iowa, Iowa City, IA, United States.

*Corresponding Author:

Prof. Thomas Graven-Nielsen, PhD, DMSc.

Center for Neuroplasticity and Pain (CNAP)

Department of Health Science and Technology

Faculty of Medicine, Aalborg University

Fredrik Bajers Vej 7 D3, DK-9220 Aalborg, Denmark

Phone: +45 9940 9832, Fax: +45 9815 4008

E-mail: tgn@hst.aau.dk

Pressure pain sensitivity (n=13)

The ANOVA results of PPTs at the TA muscle showed a significant interaction between condition*time and site*time (Fig. S1, ANOVA: F=1.41, P=0.04, P=0.015). This illustrates that the PPTs over the TA muscle were decreased in the leg injected with NGF after Day1, 2, 4, and 7 compared with PPTs at baseline (Day0) prior to injections (Fig. S1A, B, C, post-hoc: P<0.05). After correcting for multiple comparisons, no difference in PPTs were seen in the leg injected with isotonic saline when compared to baseline PPTs at Day0 (post-hoc: P>0.05). Additionally, PPTs at the middle injection site for both conditions were more decreased when compared to the PPTs at the distal site at Day1 (Fig. S1B, post-hoc: P=0.02).

The ANOVA results of PPTs on the EDL muscle showed no significant interaction between condition and time (Fig. S1D, ANOVA: F=0.43, P=0.86), nor was there a main effect for condition (post-hoc: P=0.77) or time (post-hoc: P=0.09). For the ECRB muscle, a significant interaction was seen between condition*time (Fig. S1E, ANOVA: F=2.25, P=0.05), after correction of multiple comparisons however, no difference was seen between condition (post-hoc: P=0.29) or changes over time (post-hoc: P>0.05).

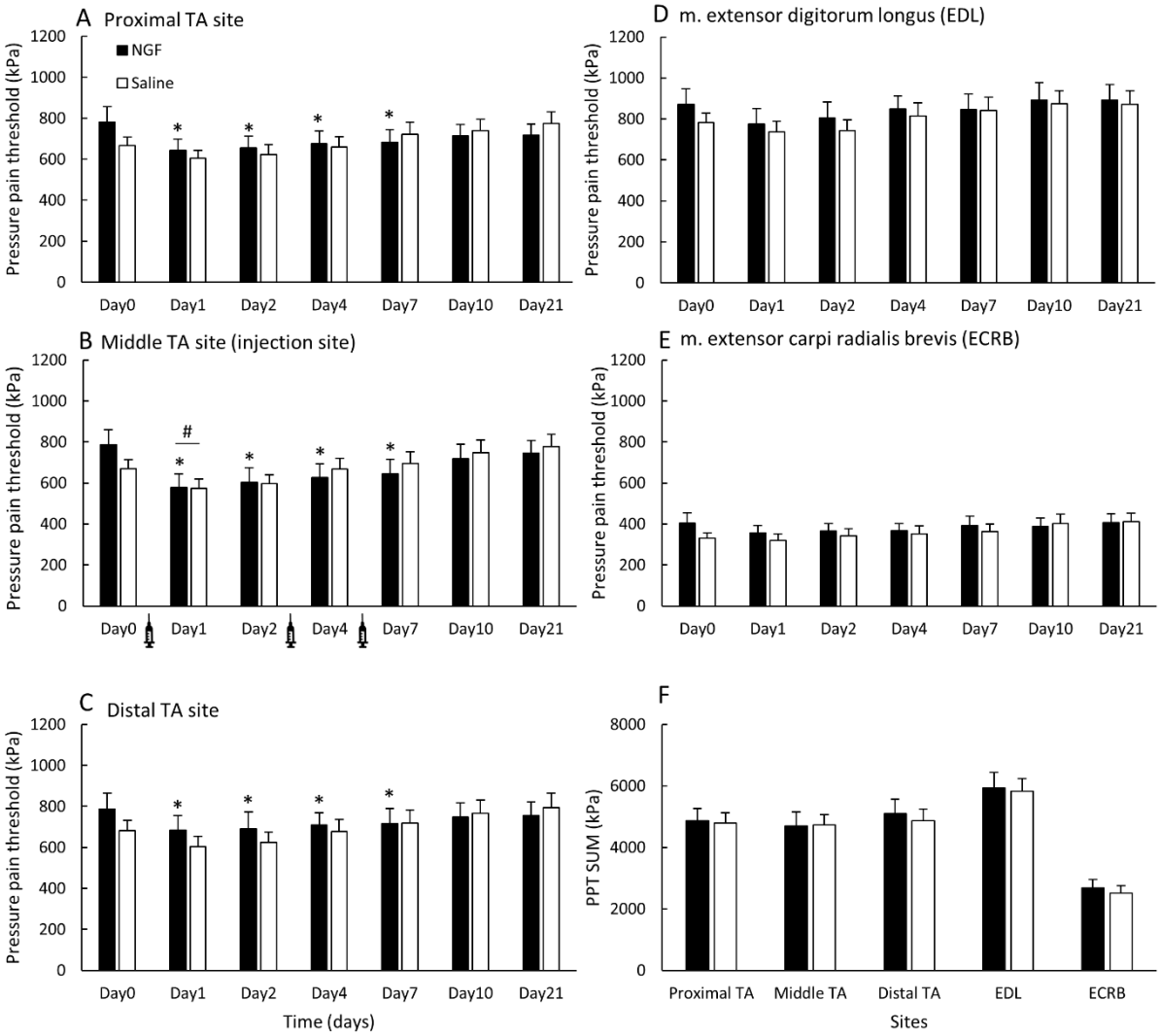

Figure S1. *Mean (+SEM, n=13) pressure pain thresholds (PPTs) following the injections of NGF (black bars) and isotonic-saline (open bars) at assessments sites over the TA muscle:* ***(A)*** *proximal site,* ***(B)*** *middle site (injection site),* ***(C)*** *distal site, and adjacent muscle:* ***(D)*** *m. extensor digitorum longus (EDL), and control site* ***(E)****: m. extensor carpi radialis brevis (ECRB). PPTs were recorded at baseline (Day0), prior to injections, and at Day1, 2, 4, 7, 10, and 21 days after injections.* ***(F)*** *Illustrates PPT-sum (sum of PPTs over time). Time of injections are indicated by the syringes at Day0, 2, and 4. Significantly different compared to Day0 (*, P<0.05). Significantly more decreased compared to PPTs at the distal site Day1 (#, P=0.02). NGF: Nerve Growth Factor, kPa: kilopascal.*

Tonic pressure-induced pain

The ANOVA results of the pain areas following the tonic-induced pressure pain at the site of injection, the middle assessment site on the tibialis anterior (TA) muscle showed no interaction between condition*time (Fig. S2, ANOVA: F=0.88, P=0.47), or any main effects in either time (P=0.64) or condition (P=0.46).

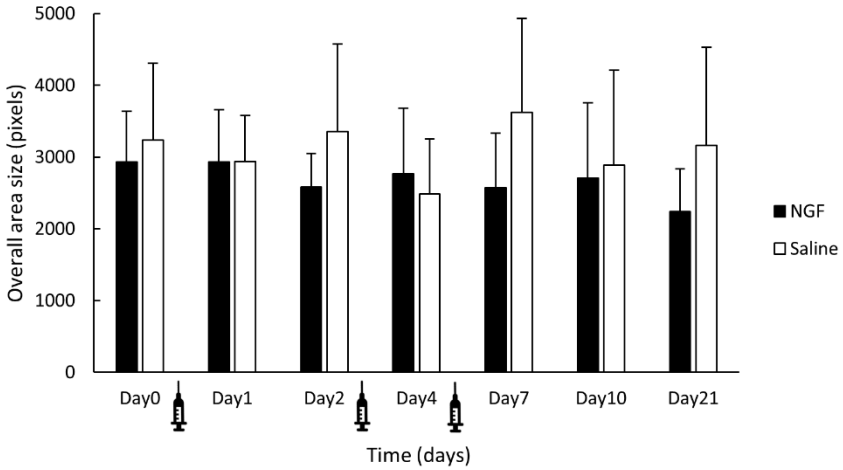

Figure S2: *Mean (+SEM, n=13) size of pain area assessed at baseline (Day0) and following days (Day1, 2, 4, 7, 10, and 21) after the injections of NGF (black bars) and control injection of isotonic-saline (white bars). Syringes indicate the time of injections. NGF: Nerve Growth Factor.*

Cuff pressure pain sensitivity

The ANOVA results of the cuff PDTs comparing the PDTs with and without the conditioning stimulus showed a 2-way interaction between conditioning*time (Fig. S3A, ANOVA: F=0.76, P=0.04), illustrating higher PDTs at all time points after the conditioning stimulus compared with the PDTs without the conditioning stimulus (post-hoc: P<0.05). There was no changes over time for either the PDTs without the conditioning stimulus (post-hoc: P>0.05), or with the conditioning stimulus (post-hoc: P>0.05). For the PTT values, comparing PTTs with and without the conditioning stimulus, the ANOVA showed a main effect of conditioning (Fig. S3B, ANOVA, F=0.78, P=0.00), indicating higher PTTs with the conditioning stimulus compared with PTTs without the conditioning stimulus (78.1±4.2 vs. 84.9±5.0).

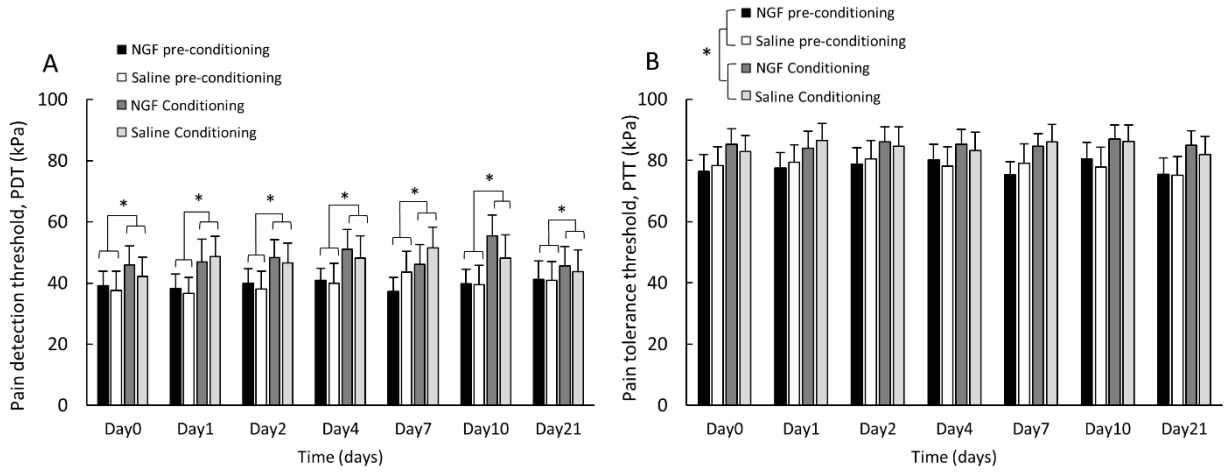

Figure S3*. Mean (±SEM, n=13) pain detection thresholds (PDTs) and pain tolerance thresholds (PTTs). PDTs* ***(A)*** *and PPTs* ***(B)*** *without conditioning after NGF (black bars) and isotonic-saline (open bars. With conditioning after NGF (dark grey) and isotonic-saline (light grey bars). Significantly different compared with PDTs and PPTs without the conditioning stimulus (*, P<0.05). NGF: Nerve Growth Factor*

Carry-over effect

Variables	Group: NGF + saline (n=6)	Group: Saline + NGF (n=7)	Carryover effect
	Sum across sessions	*Sum across session*	*Statistics (P-value)*
Likert Scale	18.5±2.2	27.1±12.9	0.55
Non-ischemic (NRS)	17.1±4.2	11.7±6.1	0.49
Ischemic (NRS)	62.4±12.8	52.6±13.6	0.62
PPT (kPa) Proximal	9294.4±901.2	9971.±11096.1	0.65
PPT (kPa) Middle	8841.4±906.2	9937.3±1234.8	0.50
PPT (kPa) Distal	9031.4±1182.4	10756.0±1185.3	0.33
PPT (kPa) EDL	10757.7±782.8	12102.7±1409.1	0.44
PPT (kPa) ECRB	4567.6±370.2	5746.4±869.9	0.26
Cuff, PDT (kPa)	443.1±93.8	645.8±98.1	0.17
Cuff; PTT (kPa)	996.4±118.1	1136.7±83.9	0.23
Pain area (Pixels)	58555.3±24581.4	24860.9±2503.0	0.17

Table S1: *Mean (±SEM, n=13) of the sum of all outcome measures across both phases (phase 1 + phase 2) within the group first receiving NGF injections and then saline (NGF+saline, n=6), and the group first receiving saline injections and then NGF (saline+NGF, n=7). NGF: Nerve Growth Factor, PPT: Pressure Pain Threshold, EDL: extensor digitorum longus, ECRB: extensor carpi radialis brevis, kPa: kilopascal.*

Variables	Group: NGF + saline (n=6)	Group: Saline + NGF (n=7)	Carryover effect
	Sum across sessions	*Sum across session*	*Statistics (P-value)*
Likert Scale	18.5±2.2	27.1±12.9	0.55
Non-ischemic (NRS)	17.1±4.2	11.7±6.1	0.49
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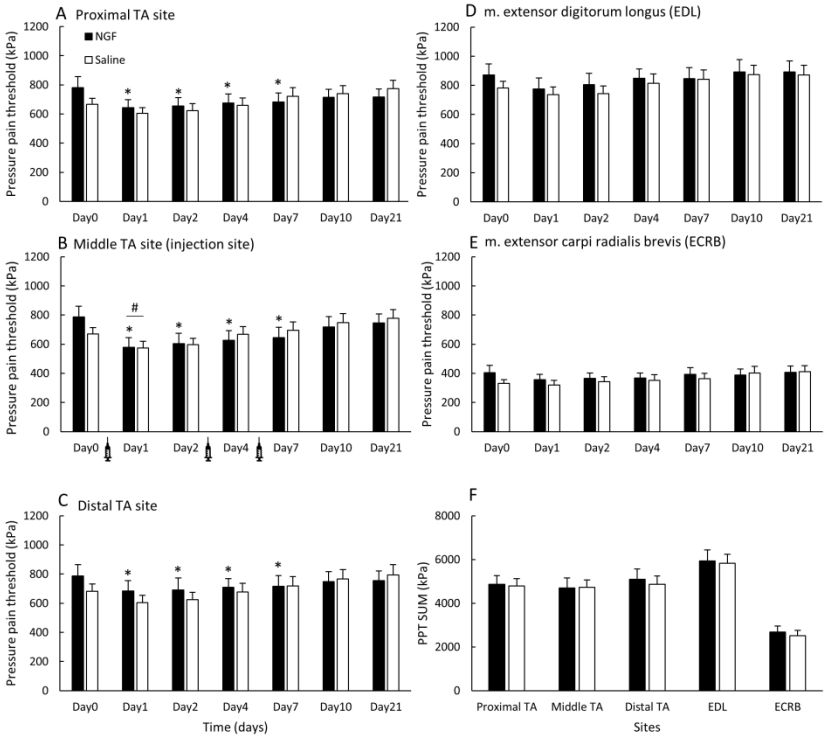

Figure S1. Mean (+SEM, n=13) pressure pain thresholds (PPTs) following the injections of NGF (black bars) and isotonic-saline (open bars) at assessments sites over the TA muscle: (A) proximal site, (B) middle site (injection site), (C) distal site, and adjacent muscle: (D) m. extensor digitorum longus (EDL), and control site (E): m. extensor carpi radialis brevis (ECRB). PPTs were recorded at baseline (Day0), prior to injections, and at Day1, 2, 4, 7, 10, and 21 days after injections. (F) Illustrates PPT-sum (sum of PPTs over time). Time of injections are indicated by the syringes at Day0, 2, and 4. Significantly different compared to Day0 (*, P<0.05). Significantly more decreased compared to PPTs at the distal site Day1 (#, P=0.02). NGF: Nerve Growth Factor, kPa: kilopascal.

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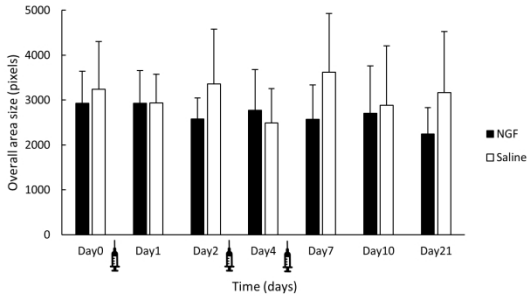

Figure S2: Mean (+SEM, n=13) size of pain area assessed at baseline (Day0) and following days (Day1, 2, 4, 7, 10, and 21) after the injections of NGF (black bars) and control injection of isotonic-saline (white bars). Syringes indicate the time of injections. NGF: Nerve Growth Factor.

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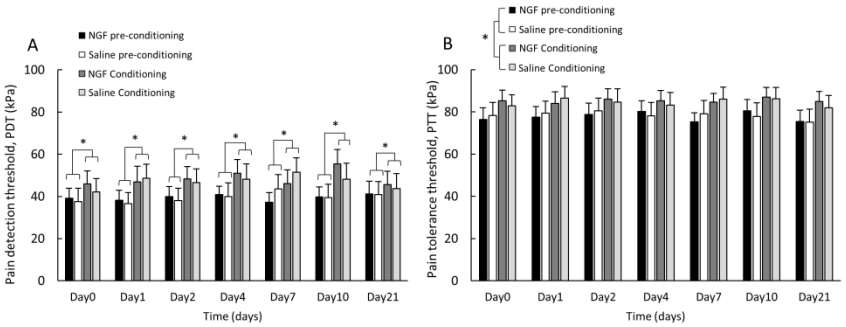

Figure S3. Mean (±SEM, n=13) pain detection thresholds (PDTs) and pain tolerance thresholds (PTTs). PDTs (A) and PPTs (B) without conditioning after NGF (black bars) and isotonic-saline (open bars. With conditioning after NGF (dark grey) and isotonic-saline (light grey bars). Significantly different compared with PDTs and PPTs without the conditioning stimulus (*, P<0.05). NGF: Nerve Growth Factor

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