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NERVE GROWTH FACTOR-INDUCED MUSCLE HYPERALGESIA FACILITATES ISCHEMIC CONTRACTION-EVOKED PAIN

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Running title: Ischemic-contraction pain following NGF sensitization

Keywords: Nerve growth factor; ischemic contraction; muscle hyperalgesia; ischemic muscle pain

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Significance

Acidification of the muscle environment may affect muscle nociceptors and pain by different mechanisms, including activation of $ASIC_3$ and TRPV1. In this study, pain evoked following ischemic contractions was increased in the NGF-sensitized muscle compared with non-ischemic contractions and in the non-sensitized muscle. These findings illustrate that responses of peripheral afferents under ischemic conditions are altered by a pre-sensitized muscle. This highlights the role of growth factors, including NGF, in peripheral muscle sensitization with clinical implications for ischemic myalgia.

ABSTRACT

Background: Intramuscular injection of Nerve Growth Factor (NGF) may influence the responsiveness of active chemo-sensitive channels affecting muscle pain sensitivity. This double-blinded crossover study in healthy humans assessed contraction-evoked pain responses and pain sensitivity during acute ischemia in the tibialis anterior (TA) muscle before and 24h after five distributed NGF injections (1µg, 4 cm interval) compared with control injections (isotonic-saline).

Methods: Twenty-one subjects participated in two experimental phases, each including 5 sessions over 7 days, with a gap of 4 weeks in-between. Muscle pain intensity evoked with daily functional tasks (Likert scale score) was collected using a paper diary. Pain intensity evoked by ischemic and non-ischemic contractions (numerical rating scale, NRS) was collected at Day0 and Day1. Pressure pain thresholds (PPTs) on the TA were recorded before (Day0), 3 hours, 1, 3, and 7 days post-injection, and after the ischemic-contractions and post-cuff deflation at Day0 and Day1.

Results: Increased Likert scores of pain were present for 7 days after NGF compared to control injections ($P<0.05$). Higher NRS pain scores of ischemic-contractions were seen when contracting the muscle injected with NGF compared to baseline ($P=0.003$) and control ($P=0.012$). Pain during non-ischemic contractions was not significantly affected by NGF injections. Decreased PPTs were found at 3 hours, Day1 and Day3 post-injection ($P<0.05$) in both conditions. Compared with pre-contractions, PPTs were increased following ischemic contractions at Day0 ($P<0.05$) and Day1 ($P<0.05$) in both conditions.

Conclusion: This study showed that ischemic contraction-evoked pain was facilitated in an NGF-sensitized muscle.

INTRODUCTION

Pain conditions, and especially clinical chronic inflammatory pain, but also experimental ischemic pain, often involve Nerve Growth Factor (NGF) driving peripheral sensitization (Mamet et al., 2003; Queme et al., 2017). In human experimental studies, single i.m. injection of NGF (5 μg) provokes time-dependent and local muscle hyperalgesia after a few hours with a peak in muscle pain sensitivity after 24-hours, returning to normal sensitivity after 7 days (Andersen et al., 2008; Nie et al., 2009; Svensson et al., 2003). Spatially distributed NGF injections of a lower dose (1 μg) into muscle tissue were recently found to provoke hyperalgesia to the same degree, but spread over larger areas compared to one bolus-injection of a higher NGF dose (Sørensen et al., 2019).

Another component present in these pain conditions involves tissue acidosis that also contributes to symptoms such as pain and hyperalgesia (Steen and Reeh, 1993). In animal studies, injection of various acidic solutions activate chemo-sensitive channels such as the acid-sensing ion channels (ASICs) and the transient receptor potential vanilloid 1 (TRPV1), with the development of mechanical hyperalgesia (Ikeuchi et al., 2008; Sluka et al., 2001). Blocking these channels by the non-specific ASIC inhibitor has further shown to attenuate the acid-induced pain, suggesting that ASICs specifically play a role in mediating the mechanical hyperalgesia (Sluka et al., 2003). In addition, experimental acidic-induced pain in humans evoke local and referred pains with an acute deep-tissue hyperalgesia in the tibialis anterior (TA) muscle during the infusion that returns to normal sensitivity after 20 min (Frey Law et al., 2008).

One channel, greatly regulated by NGF is the acid-sensing ion channel 3 ($ASIC_3$). At basal concentration, NGF is responsible for $ASIC_3$ expression in rat sensory neurons (Mamet et al., 2002), and at high concentrations, NGF increases the level of $ASIC_3$ concurrent with an increase in ASIC-like proton-mediated-currents in the dorsal root ganglion (Mamet et al., 2003). Furthermore, this channel has been suggested as the main sensor for ischemic acidosis (Benson and McCleskey, 2007). Therefore, it is plausible that an interaction may exist between NGF-sensitization and acute-induced acidic sensitivity. In a recent human study, NGF-induced hyperalgesia in the TA muscle was further facilitated at peak-pain sensitivity by a subsequent acidic infusion 24 hours after injecting NGF (Munkholm and Arendt-Nielsen, 2016). Lowering the pH level in the tissue can trigger the opening of $ASIC_3$

channels (Waldmann et al., 1999) and if availability or sensitivity of $ASIC_3$ channels is facilitated by NGF, this may explain the NGF-related facilitation of acidic-induced pain.

Acidification of the tissue environment can be induced by muscle work during anaerobic metabolism. As such, a drop in extracellular pH from 7.4 to 7.0 has been reported during ischemic contractions (Issberner et al., 1996), which is likely to open-up $ASIC_3$ channels (Birdsong et al., 2010). In line, ischemic contractions induce moderate muscle pain intensity if ischemia is maintained by a tourniquet (Mills et al., 1982). Moreover, muscle pain evoked by contractions has been observed following NGF injection (Andersen et al., 2008; Svensson et al., 2003). So far, it is unknown if ischemic contraction-evoked pain responses would be further facilitated when performing contractions in an NGF-sensitized muscle.

Assessing evoked pain responses and muscle hyperalgesia following an acute provoked acidification of the TA muscle by ischemic contractions in an NGF-sensitized muscle may clarify whether an interaction between NGF-sensitization and acute acidic-stimulation exists. In the present study, it was hypothesized that ischemic contractions in the NGF-sensitized muscle at peak NGF-sensitization (Day1), in contrast to ischemic contractions in a non-sensitized muscle at Day1 and baseline would: 1) potentiate pain-evoked responses, and 2) facilitate NGF-induced muscle hyperalgesia assessed by pressure stimulation.

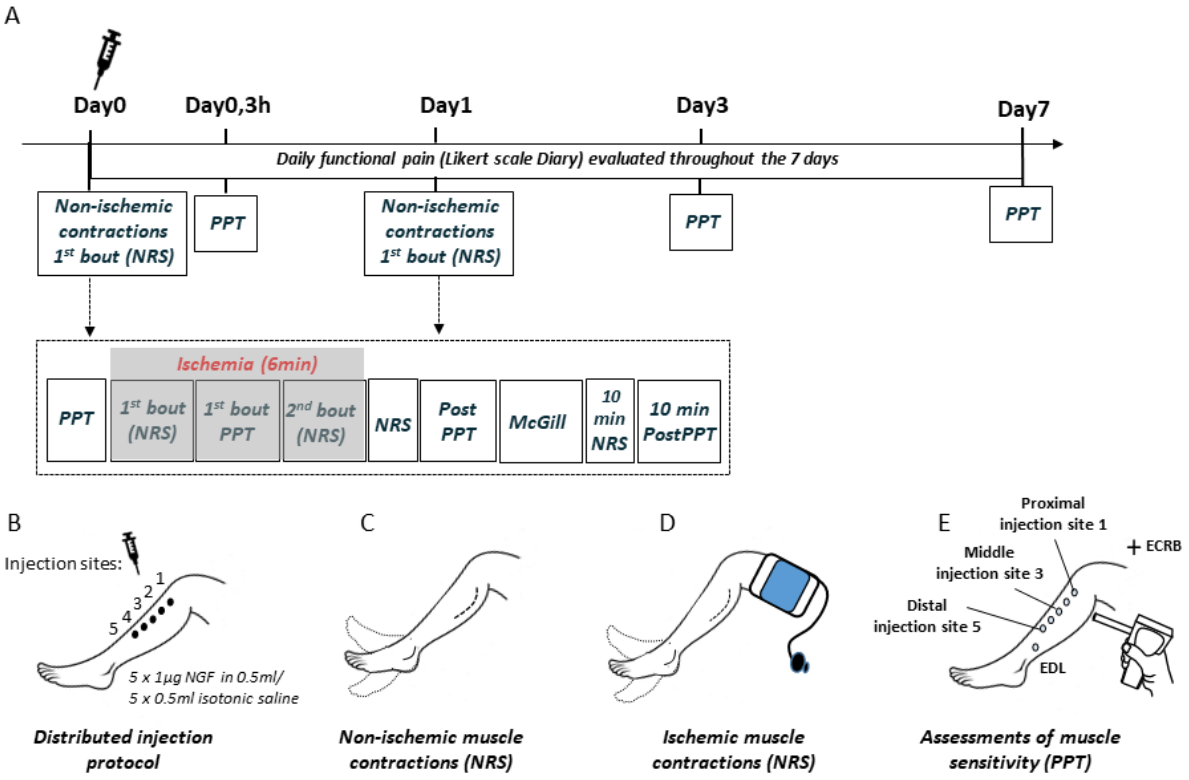
MATERIALS AND METHODS

Participants

Twenty-one healthy participants were recruited for this study through social media and advertisements at Aalborg University (mean age: 25.9 years; range: 19-35 years; seven females). No participants suffered from musculoskeletal or inflammatory conditions, or had a history of chronic pain or injuries to the lower legs within the past six month. All participants were instructed to avoid consumption of any non-steroidal anti-inflammatory drugs (NSAIDs) and to refrain from strenuous exercise of the legs throughout the study period. Prior to the first session, a verbal introduction to the study was given and written informed consent was obtained from all participants. The study was conducted in accordance with the Declaration of Helsinki (World Medical Association, 2004), approved by the local ethics committee (N-20170007), and registered at Clinicaltrials.gov (NCT0340038).

Experimental protocol

This experiment was performed as a crossover, randomized and placebo-controlled study, investigating mechanical muscle pain sensitivity and ischemic contraction-induced muscle pain before and after the muscle was sensitized by NGF or in a control condition. At the end of the first experimental session (Day0, Fig. 1A, B), the participants received five distributed injections of NGF (1µg) or five injections with isotonic saline (control) into the tibialis anterior (TA) muscle of their non-dominant leg, randomized in a balanced manner (i.e. 11 participants received NGF in the first phase). In the second phase, the type of injection not provided in the first phase was given. Before the injections at Day0 and again at Day1, an occlusion cuff was mounted proximal to the knee and inflated to occlude blood flow for 6 min, during which participants were instructed to perform a sequence of TA muscle contractions (Fig. 1D). Contraction-evoked pain intensity was recorded on a numerical rating scale (NRS) and a short-form McGill pain questionnaire (MPQ) was completed afterwards. Muscle pain sensitivity was assessed by pressure algometry before, after the first bout of ischemic contractions with maintained ischemia, immediately post and 10 min post cuff deflation (Fig. 1D). Additionally, a subgroup of participants performed repeated contractions (similar to 1st bout) in the second phase of the study before ischemia at Day0



and Day1 (Fig.1C) and rated the pain NRS scores.

Figure 1. (A) *Timeline of the five experimental sessions (Day0, Day0,3h, Day1, Day3, Day7) and assessments in each phase of the study. The ischemic condition (6 min) is shown by the gray shaded area on Day0 and Day1. Beneath, the five injection sites (1-5) within the tibialis anterior (TA) muscle are illustrated* ***(B)*** *together with the lower leg showing the non-ischemic (C) and ischemic muscle contractions* ***(D)****, and the position of the occlusion cuff mounted proximal to the knee* ***(D)****. Lastly, assessment sites for pressure algometry on the leg (proximal injection site, middle injection site, distal injection site, m. extensor digitorum longus[EDL]), and arm (extensor carpi radialis brevis [ECBR])* ***(E)****.*

The experiment included two phases with each phase divided into 5 sessions over a period of seven days: Before (Day0), 3 hours (Day0,3h) after, 1, 3, and 7 days after the injections (Fig. 1A). Four weeks interval was kept between the injections (NGF or saline) in the two phases. Each session included self-reported muscle pain with daily functional tasks (Likert scale) as well as pressure algometry in the 3h, Day3 and Day7 sessions. The self-reported muscle pain with daily functional tasks was additionally assessed in the days between sessions (i.e. 2, 4, 5, and 6 days after injections) by completing a paper dairy. The same examiner performed all assessments within the experimental procedures and was blinded to the type of injections, which were prepared and randomized by another examiner. All participants were blinded to the type of injections.

Injection protocol

The solutions of sterile recombinant human NGF were prepared by Skanderborg pharmacy, Denmark. Five injections of NGF (1 µg, 0.5 ml) were given sequentially along the TA muscle in the non-dominant leg (test phase). As a control, five injections of isotonic-saline (9 mg/ml, 0.5 ml) were given at the same sites in the opposite phase (control phase). The TA muscle and relevant landmarks were identified by manual palpation, and approximately one-third distal from the lateral femoral epicondyle on a line toward the upper edge of the lateral malleolus defined the mid-point injection site of the muscle (site 3, Fig. 1A). Four additional injection sites (site 1, 2, 4, and 5) were marked along the proximal and distal directions from the mid-point of the TA muscle with an inter-site distance of 4 cm. This protocol was based on a previously published NGF model (Sørensen et al., 2019). The injections were always given in the same order starting from the most proximal injection site (site 1) to the most distal injection site (site 5).

Daily reporting of pain with functional tasks

Subjective evaluation of muscle pain during daily function was assessed by completing a paper-based pain dairy. The evaluation was performed by use of a modified 7-point Likert scale 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' (Slater et al., 2003).

Ischemic contraction-induced pain

Ischemic muscle pain was induced in the non-dominant leg by application of a manual occlusion cuff with a handheld inflator and monometer (VBM Medizintechnik GmbH, Germany, cuff size: 107 cm/42 in.). The occlusion cuff was placed proximal to the knee and inflated to 250 mmHg to occlude arterial blood flow (Issberner et al., 1996). Before inflation, the leg was raised into a vertical position for 2 min to drain the blood. Immediately after cuff inflation, participants were instructed to perform a sequence of 45 TA muscle contractions within 90 s (1s concentric: 1 s eccentric phase). To resist dorsiflexion, a load of 3 kg, approximately 5-10 % of maximal dorsiflexion contraction effort (Stoll et al., 2000), was strapped to the distal part of the foot. Three min after the first bout of 45 contractions (1^{st} bout), five additional contractions were completed (2^{nd} bout) (Graven-Nielsen et al., 2003). The occlusion cuff was kept inflated for 6 min in total. Participants rated their pain intensity on an NRS following each bout of muscle contractions, immediately after the cuff was deflated, and again 10 min after. In the second phase of the study, a subgroup of the participants (subgroup, n=19), performed muscle contractions (45 contractions within 90 s) with and without ischemia at Day0 and Day1. For analysis, the sum of NRS scores (NRS-sum) and maximal NRS score (NRS-peak) for each session were used.

The quality of ischemic contraction-induced pain was assessed by completion of an English version of the short-form McGill pain questionnaire (MPQ) (Melzack, 1975). This included 15 words, which were numerically rated as 0 (none), 1 (mild), 2 (moderate), or 3 (severe).

Pressure pain sensitivity

Pain sensitivity to pressure was assessed at three injection sites over the TA (proximal, middle, distal), and at the extensor digitorum longus (EDL) muscles on the non-dominant leg, as well as the extensor carpi radialis brevis (ECRB) muscle on the dominant arm. The EDL muscle was identified lateral to the TA muscle by manual palpation and the assessment site was marked approximately 20 cm proximal from the upper edge of the lateral malleolus. The contralateral ECRB muscle was palpated, and included as a proximal control assessment site. A handheld pressure algometer (Somedic, Sösdala, Sweden) equipped with a 1 cm^2 circular rubber tip was used to record PPTs. Pressure was applied perpendicularly to the skin surface at each assessment site with an increment rate of 30 kPa/s. The PPT was defined as the point at which the sensation of pressure changed to the first sensation of pain. All sites were assessed three times with approximately 30 s interval between each stimulus and the average of three PPTs per site was used for further analysis.

Statistics

All data in text and figures are presented as mean and standard error of the mean (SEM) unless otherwise stated. The statistical analyses were performed using SPSS (IBM SPSS version 24), and significance level was accepted at $P \leq 0.05$. Initially, all data were checked for normality by Shapiro-Wilk test and analyzed with parametric tests when appropriate. Daily functional pain (Likert scores) and MPQ score were compared between the two conditions (NGF vs. saline) by Wilcoxon signed rank test and adjusted for multiple comparisons by Bonferroni correction. Friedman test of variance was used to compare Likert scores across time, followed by Wilcoxon signed rank tests and Bonferroni correction. The NRS-sum scores of ischemic contraction-evoked pain and NRS-peak scores were analyzed by 2-way ANOVA with the within-subject factors condition (NGF vs. saline), and day (Day0 vs. Day1). In the subgroup, NRS scores of the non-ischemic muscle contractions performed at Day0 and Day1, were analyzed by 2-way mixed model ANOVA with the between-subject factor condition (NGF vs. saline), and the within-subject factor day (Day0 vs. Day1). The NRS scores of the pain evoked by muscle contraction performed with and without ischemia were analyzed by 3-way mixed model ANOVA with the between factors: condition: (NGF vs. saline), and ischemia (with vs. without), and the within-subject factor: day (Day0 vs. Day1).

PPT values collected from the TA muscle without contractions were analyzed by 3-way repeated measures ANOVA, with the within-subject factors condition (NGF vs. saline), site (proximal, middle, distal), and time (sessions). The PPTs collected from the EDL and ECRB muscles were analyzed by 2-way repeated measures ANOVA with the within-subject factors condition and time. ANOVAs were followed by Bonferroni corrected post-hoc tests when appropriate. The sum of TA PPTs across time (without contractions) was analyzed by 2-way ANOVA with factors condition and site. The sum of PPTs from the EDL and ECBR muscle were each analyzed using a paired *t*-test.

To investigate if PPTs were affected by the ischemic contractions in the NGF-sensitized muscle, all post measures were normalized (percentages) to the pre-ischemia PPTs at the specific test day (Day0 or Day1). Any differences between Day0 and Day1 were compared between the two conditions in a 4-way repeated measures ANOVA for the TA muscle with factors condition, site, day and time (1st bout PPT, Post PPT, 10min post PPT). The EDL and ECRB muscles were analyzed by 3-way repeated ANOVA with factors condition, day and time. The sum of TA PPTs (percentages) across time (after ischemic-contractions and post cuff-deflation) was analyzed by 3-way ANOVA with factors condition and site. The sum of PPTs (percentages) from the EDL and ECBR muscle were each analyzed by a 2-way ANOVA.

To check the assumption that the period between each phase of injections ensured negligible carryover effect, a 2-step cross over trial confirmatory analysis was performed (Wellek and Blettner, 2012). This involved performing an unpaired *t*-test comparing the sum of Likert sale scores, NRS scores (NRS-sum, NRS-peak), and PPTs measured across all sessions in the two phases (NGF + saline vs. saline + NGF), followed by an additional unpaired *t*-test to test the difference between injection type across all sessions.

RESULTS

Daily reporting of pain

Compared with Day0 (baseline), Likert scores of pain evoked with daily functional tasks were increased 3 hours after the injection of NGF and remained high until Day7 (Friedman: $X^2(8)=89.9$, $P<0.00$, Wilcoxon: $P<0.05$; Fig. 2). Higher Likert scores of evoked-pain were found at all days in the NGF leg compared to the control leg (Wilcoxon, Post-hoc: $P<0.05$).

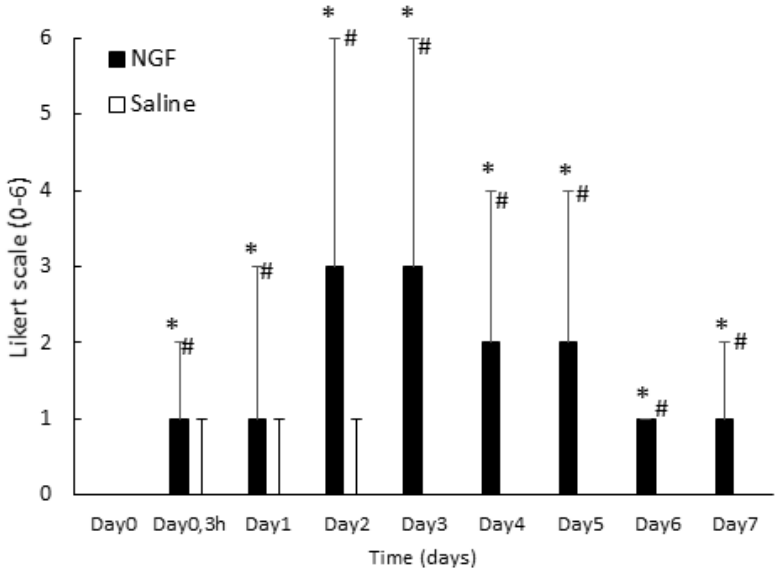


Figure 2. *Median (interquartile range, n=21) Likert scores of the pain diary for the leg injected with NGF (solid bars) and isotonic-saline (control condition, open bars). Significantly different compared with pre-injection (Day0; *, P<0.05), or compared with saline (#, P<0.05).*

Ischemic-induced contraction pain

For the NRS-sum of ischemic contraction-evoked pain, a significant interaction was found between condition and time (ANOVA: F=5.46, P=0.03; Fig. 3). Compared with the contractions performed at Day0, a higher pain NRS-sum was found at Day1 during NGF sensitization (post-hoc: P=0.003; Fig. 3). Moreover, at Day1, the pain NRS-sum was higher in the NGF sensitized leg compared with the leg injected with saline (post-hoc: P=0.01). Furthermore, the ANOVA of NRS-peak pain showed a main effect of day (ANOVA: F=3.69, P=0.01). Higher NRS-peak pain was reported following both conditions at Day1 (7.4±0.3), compared with the NRS-peak pain reported at Day0 (6.9±0.4, post-hoc: P=0.01).

The MPQ words chosen by participants to describe the ischemic contraction-evoked pain were similar for both the NGF and saline condition on Day0 before any injections were given, and at Day1 when ischemia was induced in the NGF sensitized leg or control leg (see Table S1 in supplementary material). Only the descriptor “heavy” was scored differently at Day0 between the two conditions before any injections were given (Friedman: $X^2(29)=146.7$, P<0.00, Wilcoxon: P<0.05) illustrating a higher rating for the control leg compared with the test leg (Wilcoxon: P≤0.05, Table S1, supplementary material).

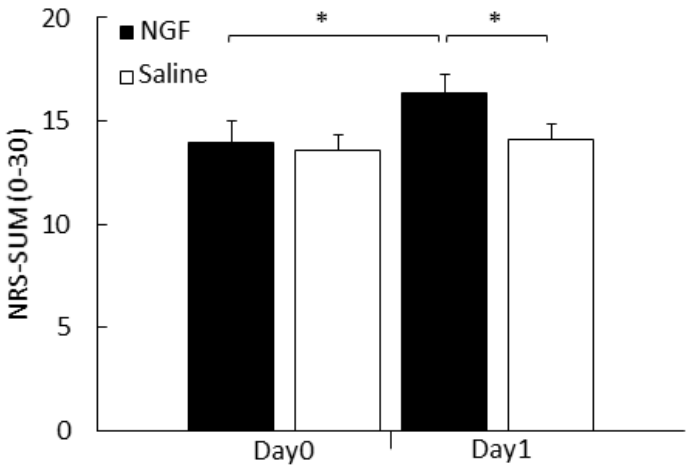


Figure 3. *Mean (±SEM, n=21) of the total sum of numerical rating scale (NRS-sum) pain scores during ischemic contractions at Day0, before NGF (solid bars) or saline (open bars) injections, and at Day1. Significantly different compared to pre-injection (Day0;*, P=0.003) and between NGF and saline at Day1 (*, P=0.012).*

Non-ischemic contractions

The NRS pain scores of contractions without ischemia were 1.7±0.3 (Day0) and 2.2±0.3 (Day1) in the group injected with NGF (n=9) and 2.1±0.5 (Day0) and 2.0±0.7 (Day1) in the saline group (n=10). The ANOVA of NRS pain scores showed no significant interaction between condition and day for the non-ischemic muscle contractions (ANOVA: F=0.718, P=0.409), nor was there a main effect for day (post-hoc: P=0.58) or condition (post-hoc: P=0.84).

Ischemic versus non-ischemic muscle contraction

The mixed ANOVA of NRS contraction pain, comparing the NRS pain scores with and without ischemia, showed a main effect of ischemia (ANOVA: F=0.377, P<0.00), illustrating that higher pain was reported when muscle contractions were performed with ischemia compared to contractions performed without ischemia (post-hoc: P<0.00, Fig. 6).

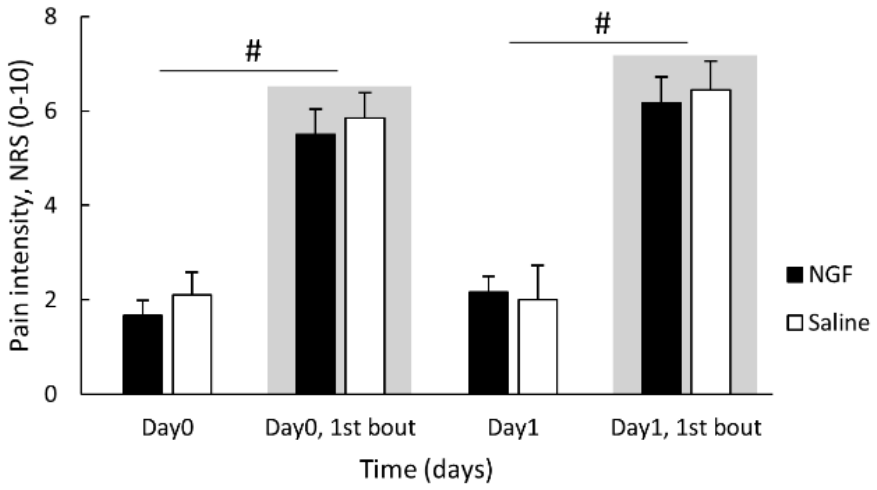


Figure 4. *Mean (± SEM) of the NRS pain score following muscle contractions performed before NGF injections at Day0 and when the leg was sensitized by NGF at Day1 (n=9, black bars), and following muscle contractions performed before saline injections at Day0, and after the injections at Day1 (n=10, control condition, open bars). The gray shaded area indicates the pain NRS scores after the 1st bout of ischemic muscle contractions. Significantly higher pain NRS scores for ischemic muscle contractions compared with non-ischemic contractions (#, P<0.00).*

Pressure pain sensitivity without contractions

The ANOVA of PPTs at the TA muscle showed a main effect of time (Fig. 4; ANOVA: F=1.02, P<0.00, Fig. 4), illustrating that PPTs over the TA muscle were lower after 3 hours, Day1, and Day3 compared with baseline (Day0) for both saline and NGF injections (post-hoc: P<0.05). Moreover, a main effect of condition indicated that the PPTs at the TA muscle were decreased even more following NGF injections compared with saline (post-hoc: P=0.05).

The ANOVA of PPTs on the EDL muscle showed an effect of time, with PPTs lower 3 hours after and at Day1 (Fig. 4D; ANOVA: F=1.13, P<0.00; post-hoc: P<0.05), and likewise for the ECRB muscle PPTs were lower at Day1 (Fig. 4E; ANOVA: F=1.59, P<0.00; post-hoc: P<0.05), when compared with baseline at Day0.

The ANOVA of the PPT-sum showed a main effect of condition for the TA muscle (Fig. 4F; ANOVA: F=0.172, P=0.05), indicating that the sum of PPTs for the leg injected with NGF was lower (i.e. more decreased) compared to the sum of PPT for the control condition. There was no difference in the sum of PPTs between the leg injected with NGF and the control leg for the EDL (t(20)=-1.192, P=0.25) or ECRB (t(20)=-0.454, P=0.65) muscles.

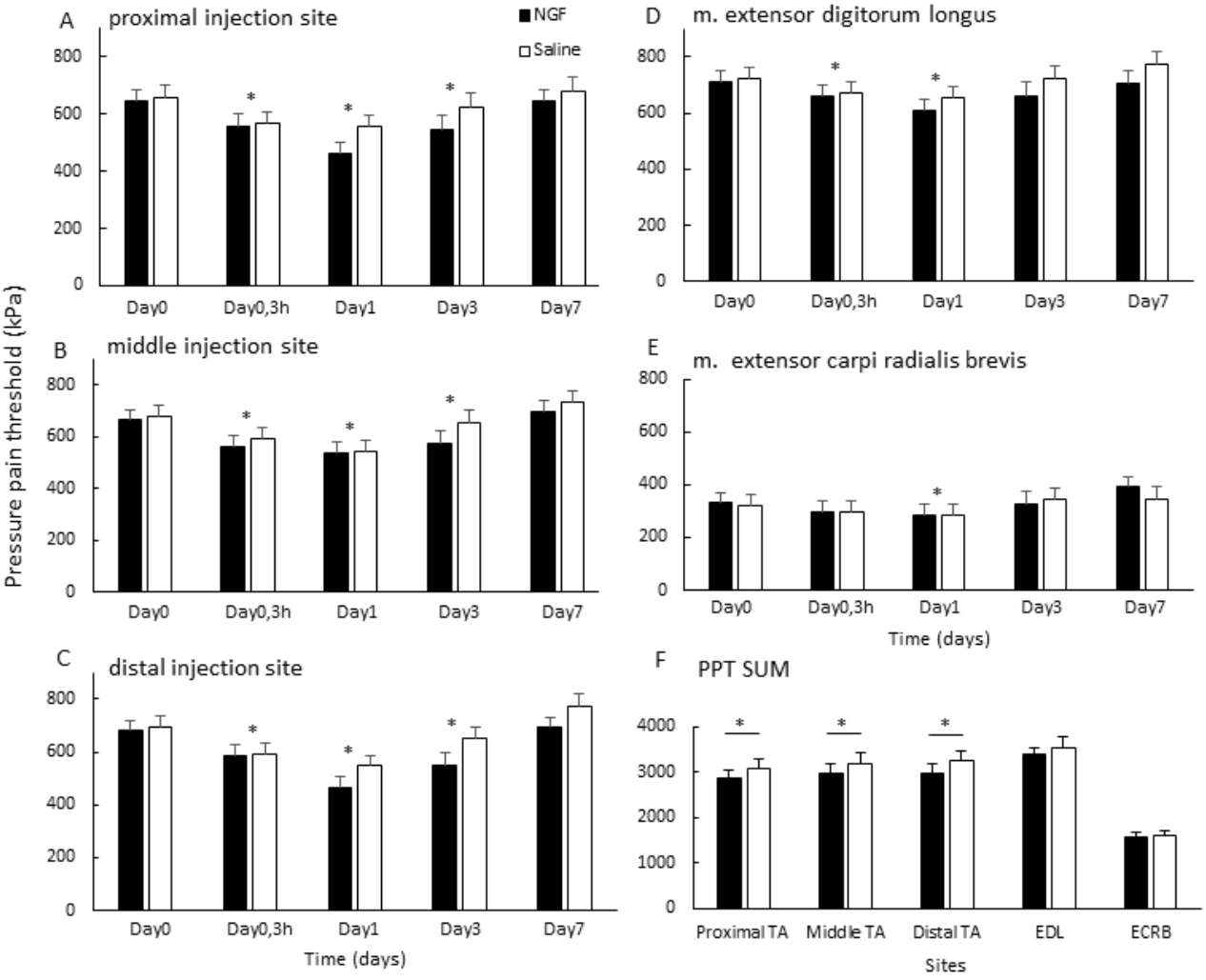


Figure 5. *Mean (+SEM, n=21) pressure pain thresholds (PPTs) for the leg injected with NGF (black bars) and saline (control, open bars) at the:* ***(A)*** *proximal injection site,* ***(B)*** *middle injection site,* ***(C)*** *distal injection site,* ***(D)*** *m. extensor digitorum longus (EDL), and,* ***(E)*** *m. extensor carpi radialis brevis (ECRB). PPTs were recorded at Day0, before the injections, at 3 hours, Day1, 3, and 7 days after injections. Significantly different compared to Day0 (*, P<0.05). Mean (+SEM, n=21) PPT-sum (sum of PPTs over time) is illustrated* ***(F)****. Significantly different compared to saline (*, P<0.05).*

Pressure pain sensitivity during and following ischemic contractions

The ANOVA of normalized PPTs recorded after the 1st bout of ischemic contraction and after cuff deflation (post PPT and 10post) on the TA muscle, showed an interaction between day and time (ANOVA: F=1.065, P<0.00; Fig. 5A, B, C). Increased PPTs were seen immediately post ischemia for both conditions at Day0 when compared to normalized PPTs measured after the 1st bout of ischemic contractions (post-hoc: P<0.05). At Day1, increased TA PPTs were seen immediately post and 10 min post ischemia for both conditions when compared to normalized PPTs after the 1st bout of contractions (post-hoc: P<0.05). Comparing the time points between Day0 and Day1, higher PPTs were seen immediately post and 10 min post ischemia on Day1 (post-hoc: P<0.05) than Day0.

The ANOVA of normalized PPTs for the EDL muscle showed an interaction between day and time (ANOVA: F=0.953, P=0.03, Fig. 5D). Increased PPTs were seen immediately post ischemia at Day1 for both conditions compared to after the 1st bout of contractions (post-hoc: P=0.005). Comparing the time points between Day0 and Day1, higher PPTs were seen immediately post ischemia (post-hoc: P=0.02), and 10 min post ischemia (post-hoc: P=0.004) at Day1 than Day0.

At the ECRB muscle, the ANOVA showed a main effect of time (ANOVA: F=1.065, P=0.00, Fig. 5E). Increased PPTs were seen immediately post and 10 min post ischemia when compared to after the 1st bout of contractions (post-hoc: P<0.05).

The ANOVA of PPT-sum showed a main effect of day for the TA muscle (Fig. 5F; ANOVA: F=1.93, P<0.00), indicating that the sum of PPTs (percentage) was higher at Day1 compared with the sum of PPTs (percentage) at Day0. The PPT-sum for the EDL muscle likewise showed a main effect of day, with a higher sum of PPTs (percentage) at Day1 compared to Day0 (ANOVA: F=0.94, P=0.01). There was no difference between days or conditions in the sum of PPTs for the ECRB muscle (ANOVA: F=1.83, P=0.1).

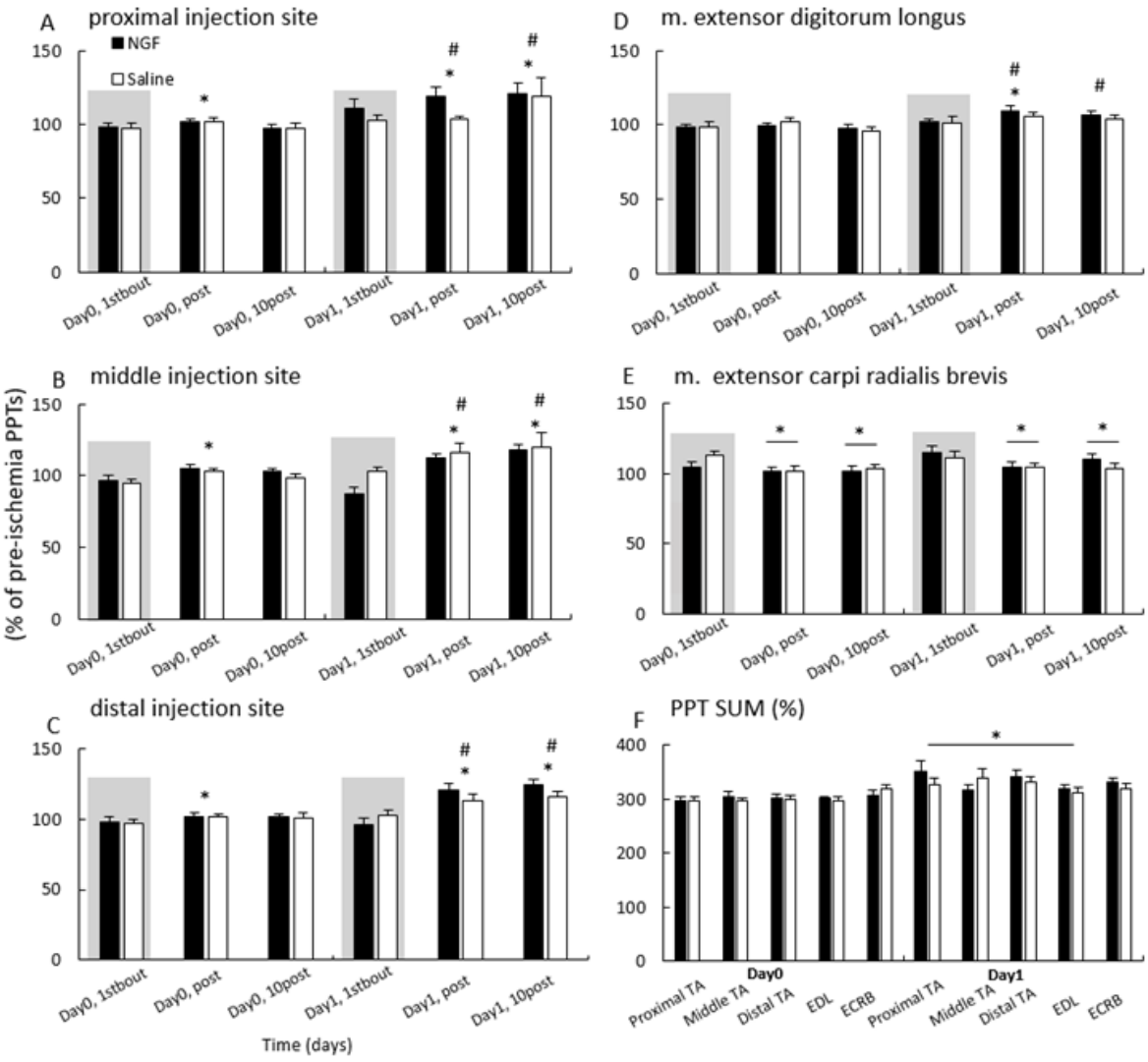


Figure 6. *Mean (+SEM, n=21) pressure pain thresholds (PPTs) normalized (%) to the first recording pre-ischemia on Day0 (before injection) and Day1 (post injection), for the leg injected with NGF (solid bars) and saline (control condition, open bars) at each assessment site: (**A**) proximal injection site, **(B)** middle injection site, **(C)** distal injection site, **(D)** m. extensor digitorum longus (**E**), m. extensor carpi radialis brevis. Normalized PPTs are illustrated after first bout of ischemic contractions (1stbout, gray shaded area), immediately after (post) and 10 min after (10post) cuff deflation on Day0 and Day1. Significantly different compared to PPTs after 1st bout of ischemic contractions (*, P<0.05). Significantly different time points compared between Day0 and Day1 (#, P<0,05). Main effect of time compared with the 1st bout of contractions (*,* P<0.05). *Mean (+SEM, n=21) of the sum of PPTs over time is illustrated for Day0 and Day1, NGF and saline conditions **(F)**. Main effect of day, significantly different from Day0 (*, P<0.05).*

Carry over effect

NRS-sum, NRS-peak, and PPTs at the middle and distal injections sites, and the EDL and ECRB muscles, did not differ in within-subjects sums of the results of both phases (NGF-saline vs. saline-NGF, P>0.05) or when comparing the injection types between the two phases (P>0.05, Table S2). This may indicate that the order of conditions (i.e. whether NGF was injected in the first phase) did not affect the outcome in the second phase of the study. In addition, the analysis showed that a higher sum of Likert scale scores was seen in the saline+NGF group compared with the NGF+saline group (t=-2.82, P=0.001). However, comparing the difference in Likert scores between the two injection types between the two phases, a higher score was seen after the NGF injections compared with saline (t=8.54, P<0.0001). A difference between injection types was also seen at the proximal injection site, showing more decreased PPTs after the NGF injections compared to saline (t=-2.214, P=0.039). Results are presented in supplementary material, Table S2.

DISCUSSION

This study showed that acute exercise-induced ischemia in an NGF sensitized muscle produced higher contraction-evoked pain than both ischemic-contractions alone and normal contractions with an NGF-sensitized muscle. Furthermore, the effect of ischemic contractions on muscle sensitivity shortly counteracted NGF-induced muscle hyperalgesia in the period after completion of the ischemic condition.

Contraction-evoked pain responses

Pain evoked by ischemic contractions and daily functional tasks, as assessed by Likert scale scores, were higher in the muscle sensitized by NGF. Higher self-perceived daily pain following intramuscular NGF injections has consistently been found in previous NGF studies when compared to control conditions (Andersen et al., 2008; Hayashi et al., 2013; Munkholm and Arendt-Nielsen, 2016). In this study, the Likert scale score summed across sessions were influenced by the previous phase, however, as this is a highly subjective measure; it could possibly be impelled by a certain expectation of NGF effect.

It is commonly known that repeated muscle contractions performed during limb occlusion produce moderate pain intensity (Graven-Nielsen et al., 2003; Newham and Mills, 2003) compared with the occlusion alone. The mechanisms of this evoked pain following ischemic contractions has been suggested to originate from both the vascular system (Eriksson et al., 1997) and from sensitized muscle nociceptors that then become responsive to the contractions (Mense et al., 2001). This is further indicated by the chosen pain descriptors from the MPQ, that include words used for both deep pain and pain that is more superficial (i.e. from skin). In addition, the quality of the overall pain experience during ischemia was closely linked to words that have previously characterized ischemic pain and did not differ between conditions (i.e. whether NGF was injected). Increased pain intensity during muscle contractions have been reported following single injections of NGF into the muscle, with a peak pain intensity around 3 to 3.5/10 on a visual analogue scale (VAS) (Andersen et al., 2008; Bergin et al., 2015; Nie et al., 2009), and a peak pain at 4/10 on a VAS when distributing the NGF injections (Sørensen et al., 2019). In contrast, peak pain evoked by ischemic contractions alone has been reported as 6.4/10 VAS (Graven-Nielsen et al., 2003). In the present study, a peak pain intensity of 6.7/10 NRS was reported for ischemic contractions alone before any injections were given; whereas a peak pain intensity of 7.4/10 NRS was reported for ischemic contractions of both the NGF sensitized leg and control. The overall pain intensity (NRS-sum) was higher during NGF sensitization compared with the control condition, suggesting that the pain evoked by ischemic contractions may be facilitated when NGF has sensitized the muscle.

NGF-induced muscle hypersensitivity

Muscle hyperalgesia developed 3 hours after the injections and lasted until Day3 in both conditions, with more pronounced hyperalgesia following the injections of NGF. This timeline of muscle hyperalgesia is a consistent finding among previous human NGF studies following single injection of 5µg NGF (Andersen et al., 2008; Nie et al., 2009). The present study shows that a washout period of four weeks between the injections is sufficient to avoid carryover effects in PPT measures, indicating that the decrease in PPTs in the control condition (saline injections) are most likely not affected by previous injections of NGF. As NGF shows a peak effect approximately 1-2 days after injection, it could be speculated that the decreased PPTs in the control condition 3 hours after, could be driven by a placebo

effect (Frisaldi et al., 2017). Additionally, as NGF is upregulated in response to unaccustomed and strenuous muscle contraction (Murase et al., 2010), it cannot be ruled out, that a potential release of NGF would account for the muscle hyperalgesia seen at Day1- Day3 in the current control phase.

Decreased PPTs were also seen at the EDL assessment site 3 hours after and at Day1, which suggested to reflect a widespread effect of NGF (Andersen et al., 2008). However, this was not observed after distributing the NGF injections along the TA muscle (Sørensen et al., 2019), making it more likely that the current finding results from the involvement of the ankle joint during the dorsiflexion and, hence affecting the EDL muscle during the contraction task. Although an effect on muscle sensitivity was seen at the ECRB muscle at Day1, control sites located extra segmentally seem not to be affected by NGF (Schabrun et al., 2016).

The sum of PPTs over time showed that the leg injected with NGF was more decreased compared with the sum of PPTs for the saline injections, suggesting that NGF induced an overall mechanical muscle hyperalgesia when compared with the control condition.

Ischemic effects on muscle pain sensitivity

In this study, decreased muscle pain sensitivity to pressure stimulation was seen immediately after and up to 10 min after completion of the ischemic contractions at post cuff deflation at Day1 in both conditions. This indicates that the acute ischemic environment in the muscle did not have any facilitating effect on muscle hyperalgesia. Munkholm et al. (Munkholm et al., 2016) showed that an infusion of acidic saline (pH 5.4) 1 day after NGF injection further decreased PPTs locally at the site of injection in the sensitized TA muscle, and maintained this exacerbated muscle hyperalgesia until Day2. In contrast, the acidic saline did not cause any changes in muscle sensitivity assessed at a proximal TA site or in the control muscle injected with isotonic saline. In support of such findings, injection of acidic saline in mice has been shown to produce local mechanical hyperalgesia (Sluka et al., 2001), that further can be attenuated by specifically targeting $ASIC_3$ receptors (Sluka et al., 2003). In the current study, it was speculated that the $ASIC_3$ and TRPV1 channels could be activated as result of a general influence on the muscle milieu i.e. by lowering the pH level locally within the TA compartment via ischemia (Birdsong et al., 2010). Based on the plausible assumption that pH did change in the present study, the provoked acidification of the

muscle environment might not have any facilitating effect on muscle sensitivity. If the lowering of pH was sufficient to activate these chemo-sensitive channels in this study, such a small drop might not have been enough to either activate or further sensitize the muscle nociceptors after NGF injections, as was seen following acidic infusions in the study by Munkholm et al. (Munkholm and Arendt-Nielsen, 2016). In an animal study, Steen et al. (Steen et al., 1992) showed that the threshold levels for activating nociceptors by acid buffers ranged from pH 6.9 to 6.1, with a maximum discharge at pH 5.2. Therefore, the pH sensitivity on nociceptors may play an important role in developing muscle hyperalgesia, suggesting however, that other mechanisms such as exercise or movement, may account for the decrease in muscle sensitivity seen after the ischemic condition in this study.

A study from 2003 reported a short-lasting decrease in muscle sensitivity during pain evoked by ischemic contractions (Graven-Nielsen et al., 2003). Afferent inhibition could possibly be a reason for the increase in PPTs during such contraction task (Kosek and Lundberg, 2003). Additionally, decreased sensitivity to pain is a common finding due to exercise (Naugle et al., 2013), and has further been observed immediately after the performance of aerobic (Naugle et al., 2013; Vaegter et al., 2018) and isometric exercise (Vaegter et al., 2019), and up to 5 min in a muscle following the performance of an isometric contraction task of the leg (Kosek and Ekholm, 1995). In the current study, the increase in PPTs occurred immediately after cuff deflation at pre-injection, and this was more increased at Day 1 post injection, in the NGF sensitized muscle, immediately after and up to 10 min after completion of the ischemic contractions after cuff deflation. Although different mechanisms may be implicated, this contrasts the findings by Lannersten (Lannersten and Kosek, 2010) in which isometric contractions of an affected muscle (i.e. painful muscle) in a group of myalgia patients increased the muscle sensitivity up to 10 min after the task, compared to a decrease in sensitivity at a distant and non-painful muscle. They suggested this was because of dysfunctional endogenous pain inhibition. Based on this, the change in muscle sensitivity observed after ischemic contractions in this study might be explained by normal inhibition during pain evoked by the exercise-induced ischemic contractions that shortly counteracts or masks the established NGF-induced muscle hyperalgesia.

Conclusion

An acute provoked acidification of the TA muscle environment by exercise-induced ischemia does not facilitate NGF-induced muscle hyperalgesia, but may sensitize the muscle nociceptors by different mechanisms, including the activation of chemo-sensitive channels, since pain evoked by ischemic contractions was higher in NGF sensitized muscle compared to ischemic contractions alone. This interaction between NGF-sensitization and acidic stimulation may play an important role in peripheral muscle sensitization with clinical implication in ischemic pain conditions.

Author Contributions

All authors were involved in the design and structuring of the study. LBS performed the experiments and collected the data. TGN and LBS analyzed the data. All authors contributed in writing and revisions and reviewed versions of the manuscript and all discussed the results and commented on the final version.

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Clinical Study Registration

The study was registered at Clinicaltrials.gov (NCT0340038).

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Figure legends

Figure 1. (A) Timeline of the five experimental sessions (Day0, Day0,3h, Day1, Day3, Day7) and assessments in each phase of the study. The ischemic condition (6 min) is shown by the gray shaded area on Day0 and Day1. Beneath, the five injection sites (1-5) within the tibialis anterior (TA) muscle are illustrated **(B)** together with the lower leg showing the non-ischemic (C) and ischemic muscle contractions **(D)**, and the position of the occlusion cuff mounted proximal to the knee **(D)**. Lastly, assessment sites for pressure algometry on the leg (proximal injection site, middle injection site, distal injection site, m. extensor digitorum longus[EDL]), and arm (extensor carpi radialis brevis [ECBR]) **(E)**.

Figure 2. Median (interquartile range, n=21) Likert scores of the pain diary for the leg injected with NGF (solid bars) and isotonic-saline (control condition, open bars). Significantly different compared with pre-injection (Day0; *, $P<0.05$), or compared with saline (#, $P<0.05$).

Figure 3. Mean (±SEM, n=21) of the total sum of numerical rating scale (NRS-sum) pain scores during ischemic contractions at Day0, before NGF (solid bars) or saline (open bars) injections, and at Day1. Significantly different compared to pre-injection (Day0;*, $P=0.003$) and between NGF and
saline at Day1 (*, $P=0.012$).

Figure 4. Mean (± SEM) of the NRS pain score following muscle contractions performed before NGF injections at Day0 and when the leg was sensitized by NGF at Day1 (n=9, black bars), and following muscle contractions performed before saline injections at Day0, and after the injections at Day1 (n=10, control condition, open bars). The gray shaded area indicates the pain NRS scores after the 1st bout of ischemic muscle contractions. Significantly higher pain NRS scores for ischemic muscle contractions compared with non-ischemic contractions (#, $P<0.00$).

Figure 5. Mean (+SEM, n=21) pressure pain thresholds (PPTs) for the leg injected with NGF (black bars) and saline (control, open bars) at the: **(A)** proximal injection site, **(B)** middle injection site, **(C)** distal injection site, **(D)** m. extensor digitorum longus (EDL), and, **(E)** m. extensor carpi radialis brevis (ECRB). PPTs were recorded at Day0, before the injections, at 3

hours, Day1, 3, and 7 days after injections. Significantly different compared to Day0 (*, P<0.05). Mean (+SEM, n=21) PPT-sum (sum of PPTs over time) is illustrated **(F)**. Significantly different compared to saline (*, P<0.05).

Figure 6. Mean (+SEM, n=21) pressure pain thresholds (PPTs) normalized (%) to the first recording pre-ischemia on Day0 (before injection) and Day1 (post injection), for the leg injected with NGF (solid bars) and saline (control condition, open bars) at each assessment site: (**A**) proximal injection site, **(B)** middle injection site, **(C)** distal injection site, **(D)** m. extensor digitorum longus (**E**), m. extensor carpi radialis brevis. Normalized PPTs are illustrated after first bout of ischemic contractions (1stbout), immediately after (post) and 10 min after (10post) cuff deflation on Day0 and Day1. Significantly different compared to PPTs after 1^{st} bout of ischemic contractions (*, P<0.05). Significantly different time points compared between Day0 and Day1 (#, P<0,05). Main effect of time compared with the 1^{st} bout of contractions (*, P<0.05). Mean (+SEM, n=21) of the sum of PPTs over time is illustrated for Day0 and Day1, NGF and saline conditions **(F)**. Main effect of day, significantly different from Day0 (*, P<0.05).