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ENLARGED AREAS OF PAIN AND PRESSURE HYPERSENSITIVITY BY SPATIALLY DISTRIBUTED INTRAMUSCULAR INJECTIONS OF LOW-DOSE NERVE GROWTH FACTOR

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Disclosures

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Highlights

- Distributed low-dose NGF injections induce pronounced muscle hypersensitivity and enlarged pain areas evoked by muscle contraction
- Distributed NGF injections affect a larger area of the muscle compared to single bolus NGF
- Low-dose NGF sensitize muscle nociceptors locally
- Distributed NGF-injection protocol may mimic clinical muscle pain better as the entire muscle is affected and be relevant for studies of prolonged muscle pain.

ABSTRACT

Intramuscular injection of nerve growth factor (NGF) causes muscle hyperalgesia without immediate pain. This double-blinded, randomized study assessed pain and muscle hypersensitivity after a single-site bolus NGF injection (5µg) compared with five spatially distributed, low-dose NGF injections (1µg, 4cm distance) into the tibialis anterior (TA) muscles in 20 healthy subjects. Injection-pain was rated on a visual analogue scale (VAS). Reports of muscle pain with functional tasks (Likert scale score) and presence of spontaneous pain were collected daily using a diary. Pressure pain threshold (PPTs), overall pain intensity (numerical rating scale, NRS) and pain areas following TA contraction were collected at baseline, 3 hours, 1, 3, 7, 14, and 21 days post-injection. Low immediate VAS-scores were associated with both injection protocols. Likert scores showed moderate pain intensities, but no spontaneous pain, until Day12 for both injection-protocols ($P<0.05$). Reduced PPTs at the 5µg and 1µg injection sites were found after 3 hours lasting until Day7 ($P<0.05$). The 1µg injection provoked decreased PPTs at Day1 ($P=0.036$) at proximal injection-site, and at Day1 ($P=0.02$) and Day3 ($P=0.01$) at distal injection-site. TA muscle contraction resulted in larger pain areas and higher NRS scores at Day3 for the distributed injections compared with the single-site injection ($P<0.001$).

Perspectives

Spatially distributed low-dose NGF injections induced prolonged pain, mechanical muscle hypersensitivity and enlarged contraction-evoked pain areas. These features mirror some clinical muscle pain conditions where diffuse pain areas and muscle hypersensitivity is present during daily activities. Low-dose NGF injections may be useful for further studies of prolonged pain conditions.

Keywords: Nerve growth factor, hyperalgesia, pain measurements, injection, muscle contraction

INTRODUCTION

Nerve growth factor (NGF) is a neurotrophic factor involved in pain sensitization [22] and associated with chronic pain conditions [20]. Elevated NGF levels have been found in the cerebrospinal fluid of patients with chronic headache and fibromyalgia [25], and linked to increased pain intensity in patients with an inflammatory condition [22]. An early study in healthy volunteers and patients assessing the therapeutic potential of NGF showed that the dose-limiting effect of NGF was pain and hyperalgesia at the site of injection [2]. Moreover, mild to moderate muscle pain was reported several hours after intravenous (i.v) administration of larger NGF doses (0.03 to 1 µg/kg) [17,21], suggesting that the larger i.v doses reaches the sensory neurons in a widespread manner and at a concentration adequate to excite the nociceptors and elicit pain. Even though NGF directly excites nociceptors [11], pain has not been evoked immediately after intramuscular (i.m) injection or further, reported as spontaneous pain, in the days following the injection [1,12,19]. Compared with a widespread distribution of NGF following i.v delivery, a bolus injection locally deposited into the muscle tissue may exceed what is needed for exciting or further sensitizing available nociceptors. Therefore, the absence of pain may be due to a complete excitation or sensitizing effect on nociceptors locally to the injection although not sufficient for inducing pain [13,15], and the excess NGF accumulating in the tissue would have no further effect. Hence, a bolus injection might not be adequate for inducing local pain.

Peripheral and central mechanisms may account for NGF-induced muscle hyperalgesia observed at the injection-site, whereas widespread sensitizing effects may be centrally mediated [9,15]. Previous human studies demonstrated that 5µg NGF injected intramuscularly induces a time-dependent and local hyperalgesia 3 hours after administration with a maximum decrease in pressure pain thresholds (PPTs) at day 1, lasting up until day7 [1,19]. Areas of hyperalgesia after bolus injection of NGF into the tibialis anterior (TA) muscle expanded proximally and distally from the injection site one day after the injection [1]. Daily injections of NGF (5µg) to the same site on 3 consecutive days prolonged the duration of the hyperalgesia up to 10 days [12,26] without further reduction in PPTs. These results suggest that reduced PPTs were maintained by the daily

injections, but the extent of the reduction was non-cumulative or saturated.

Central mechanisms, namely sensitization, has been suggested to underlie the findings of enlarged pain areas provoked by tonic painful pressure stimulation to the muscle that further expanded 24 hours thereafter [6]. Similarly, expanded pain areas following tonic painful pressure stimulation developed progressively with 3 repeated daily NGF injections, which has not yet been shown in studies using single NGF administration [12]. Assessing pain and hyperalgesia during functional tasks, rather than at rest, may provide further insight into the characteristics of NGF-induced prolonged muscle pain that can mimic some aspects of clinical muscle pain conditions. Intramuscular injection of NGF evokes pain with strenuous contraction of the jaw muscle after 1 day lasting up to 7 days [29] and by moderate contractions of the TA muscle 3 hours after the injection lasting up until day 7 [1]. It is unknown, however, whether contraction-evoked hyperalgesia would be more pronounced if NGF was administered over a larger part of the muscle by distributed injections.

In the present study, it was hypothesized that spatially distributed injections of low-dose NGF, in contrast to a single-site bolus injection of NGF (same total dose), into the TA muscle would: 1) cause immediate and spontaneous pain, 2) sensitize a larger area of the muscle, which over time cause a higher pain intensity evoked by muscle contraction, and 3) cause a larger area of muscle hyperalgesia assessed by pressure stimulation.

MATERIALS AND METHODS

Participants

Twenty healthy subjects were recruited through social media and advertisements at Aalborg University (mean age: 24.7 years; range: 21-35 years; 10 females). The subjects had body mass indices within the normal range (22.5 kg/m^2; range 17.6-26.8 kg/m^2) and none of the subjects had any pain complaints or history of injuries to the lower legs within the past six months. Prior to the first experimental day, all subjects took part in a training session in order to be familiarized with the testing procedure (no injections). The subjects were additionally instructed not to take any non-steroidal anti-inflammatory drugs (NSAIDs) and to refrain from any strenuous leg exercise

causing muscle soreness throughout the entire study period. All subjects were given a verbal introduction to the study procedures and written informed consent was obtained prior to the first session. The study was approved by the local ethics committee (N-20170007), registered at Clinicaltrials.gov (NCT03217942), and conducted in accordance with the Declaration of Helsinki [23].

Experimental protocol

The study was designed as a randomized, double-blind, and controlled follow-up experiment investigating the time-course (0-21 days) and distribution of mechanical muscle hyperalgesia and pain responses following two NGF injection protocols (low-dose distributed, single-site bolus) in the TA muscles. After baseline assessment on Day0, each subject received both NGF injection protocols, one in each leg. The sequence of legs receiving either the single-site bolus NGF injection protocol or the distributed injections of NGF was randomized and balanced between subjects (i.e., 10 subjects had the right TA muscle injected with the single-site bolus NGF injection). Five injection sites were identified by manual palpation and marked along the TA muscles (Fig. 1). The bolus injection protocol included one injection of NGF (5µg) into the midpoint injection site (site 3) and the four remaining sites received control injections of isotonic saline to ensure blinding. The spatially distributed protocol consisted of five low-dose injections of NGF (1µg) injected sequentially along the TA muscle. The pain intensity was recorded continuously during all injections.

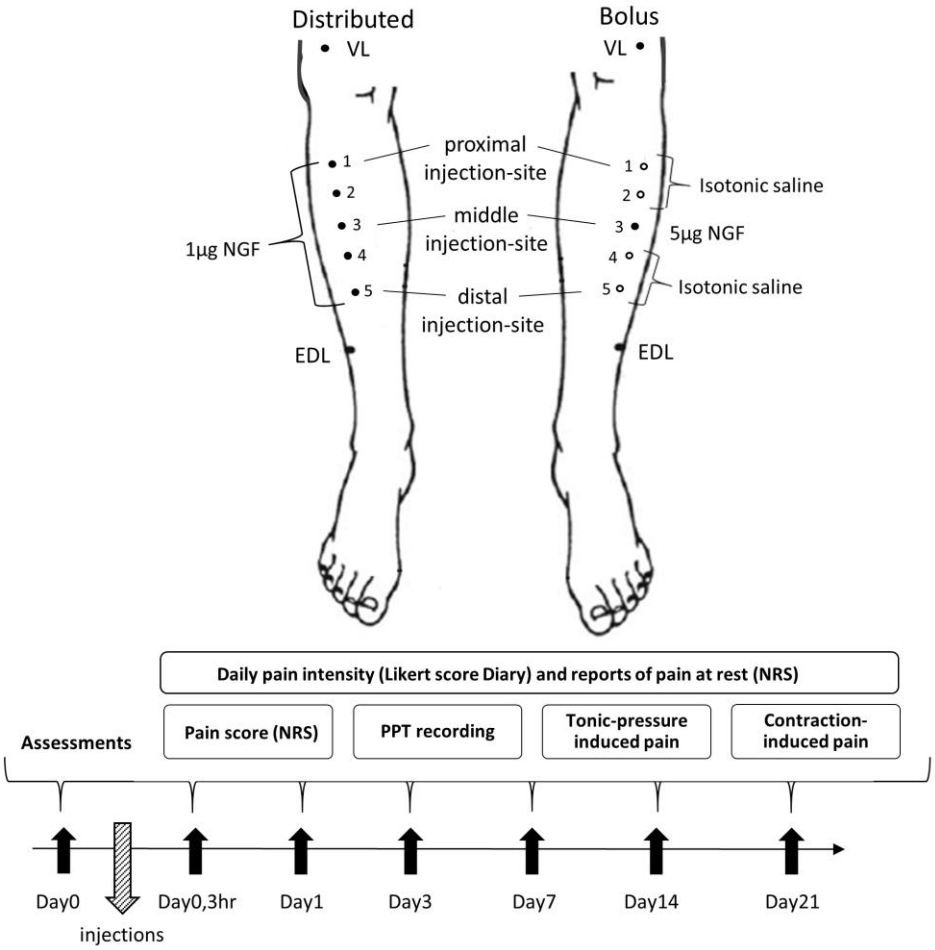

Fig. 1 A) Illustration of the five injection sites (1, 2, 3, 4, 5) within the tibialis anterior muscles (TA) for each injection protocol, and assessment sites for mechanical pressure stimulation (proximal injection site, middle injection site, distal injection site, m. extensor digitorum longus/EDL, m. vastus lateralis/VL). **B)** Experimental timeline of the seven assessment sessions and the assessment protocol.

The study was divided into seven sessions over a period of 21 days: before (baseline, Day0), 3 hours (Day0), 1, 3, 7, 14, and 21 days after the injections (Fig. 1). Each session included a verbal rating of spontaneous pain (NRS) determined as muscle pain at rest, assessment of mechanical pain sensitivity by pressure algometry, contraction-induced muscle pain following a dorsiflexion

task, tonic pressure-induced pain, and pain drawings. Self-reported muscle pain during daily functional tasks was assessed for 21 days by completing a Likert scale pain dairy. The same examiner performed all assessments and experimental procedures, while another examiner prepared and randomized the injections. All subjects were blinded with respect to the injection protocol of NGF.

NGF injection protocols

The single-site bolus injection protocol included one injection of recombinant human NGF (5µg, 0.5 ml, Skanderborg pharmacy, Denmark) injected into the midpoint injection site of TA [1,18] and 4 injections of isotonic saline (9 mg/ml, 0.5 ml) distributed into the proximal and distal injection sites (Fig. 1; sites 1, 2, 4, and 5). In the contralateral leg, recombinant human NGF (1µg, 0.5 ml, Skanderborg pharmacy, Denmark) was injected into all five injections sites. All injections were given manually, and each individual injection was completed over approximately 10 s with a 10 s

interval between injections. The order of injections was not randomized, but in both protocols, the midpoint injection site was always injected first and then injections were given alternating between proximal and distal sites. The mid-point of the TA muscle belly defined the middle injection site (site 3) and was located approximately one-third distal from the lateral femoral epicondyle down to the upper edge of the lateral malleolus (Fig. 1). The injection sites at the TA belly were marked at a 2 cm distance lateral to the tibial bone. As i.m injection of isotonic saline only spreads a few centimeter from the injection area in the TA muscle [11], it was assumed that the NGF solution would stay relatively localized and not spread to the neighboring injection sites. Hence, the two distal and proximal injection sites (site 1, 2, 4 and, 5), were marked with respect to the midpoint injection site along the TA muscle with an inter-site distance of 4 cm.

Assessment of pain intensity during injection

During the five injections, subjects rated the intensity of pain continuously for 5 min for each injection-protocol on a visual analogue scale (VAS) using a tablet (VAS app; Aalborg University) displaying a 10 cm line with the anchors ‘no pain=0’ and ‘worst pain imaginable=10’. The VAS data were sampled with a frequency of 1 Hz to reflect the temporal pain profile of each injection-

protocol. Mean VAS score sampled in the period during the injection procedure and the two periods post injections (Fig. 2) as well as the area under the VAS-time curve (VAS-area) were extracted for both the distributed NGF injections and the single-site bolus NGF protocol.

Pressure pain sensitivity

PPTs were assessed at three injection sites (Fig.1; most proximal, middle, and most distal) and at the extensor digitorum longus (EDL) and vastus lateralis (VL) muscles. The site on the EDL muscle was identified lateral to the TA muscle by manual palpation and approximately 20 cm in a straight line proximal from the upper edge of the lateral malleolus. The EDL muscle assessment site was included as this is innervated by the deep peroneal nerve common to the injected TA muscle [1]. The VL assessment site was included as a proximal control site. All sites were marked with a semi-permanent marker, and subjects kept the sites visible by re-applying the marker after bathing.

PPTs were recorded with a handheld pressure algometer (Somedic, Hörby, Sweden) equipped with a standard circular footplate of 1 cm^2. Pressure was applied perpendicularly to the skin overlying muscles of interest with an increment rate of 30 kPa/s. The subjects were instructed to press a stop button when the sensation of pressure changed to pain. All sites were assessed three times and the pressure stimulation was given separately at each site alternating between right and left leg with a 30 s interval. The average PPT of the three assessments for each site was used for statistical analysis.

Tonic pressure-induced pain

To evaluate the effect of supra-threshold pressure pain stimulations, a 30-s tonic pressure stimulation at 120% of the PPT recorded in the respective session was used [6]. Supra-threshold pressure was applied using the same handheld pressure algometer (Somedic, Hörby, Sweden) with an ultrashort ramp to reach the 120% stimulation intensity within few seconds of stimulation before this was held constant for 30 seconds. To test for a possible change in central mechanisms linked with pain referral [6] the subjects were asked to draw areas of pressure-induced pain following the tonic stimulation on a digital body chart as shown on a tablet (NavigatePain, Denmark). The distal to proximal length, medial to lateral width of the pain area, overall area and

area under the curve (AUC) were extracted from the program and used to analyze local extension of the tonic pressure-induced pain.

Contraction-induced muscle pain

Subjects were asked to perform a simple contraction task with their lower leg [1]. During the task, subjects sat on a bed with their legs hanging over the edge such that their knees flexed to approximately 90°. The subjects contracted the TA muscle by performing a dorsiflexion by moving the foot from a flexed position to a fully extended position, while keeping the foot slightly inverted. The simple contraction task was repeated 10 times for each foot. The task was performed slowly but in a self-chosen speed and subjects were encouraged to use the same speed throughout the experiment. Subsequently, the subjects verbally rated the overall pain intensity when performing the 10 muscle contractions using a numerical rating scale (NRS) with the anchors of 0 for 'no pain' and 10 for 'worst pain imaginable pain. After this, the subjects were asked to draw the area of contraction-induced pain on a digital body chart (Navigate Pain App, Aalborg University, Denmark). The distal to proximal length, medial to lateral width of the pain area, overall area, and area under the curve (AUC) of the time vs pain-area relation were extracted, and used to analyze the extent of the contraction-induced pain.

Daily reporting of pain with functional tasks

Subjective evaluation of muscle pain during daily function was assessed in the morning and in the evening throughout the 21 day study period by filling out a paper diary. The daily pain was evaluated using 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' [27]. Finally, subjects were asked if they felt any spontaneous unprovoked pain. Daily functional pain intensity was calculated as a mean

score of the morning and evening Likert scores, averaged across 4 days (Days 1-4, 5-8, etc.) and used for analysis of peak and mean Likert scores across the time points.

Statistics

Data are presented as mean ± standard error of the mean (SEM) in text and figures unless otherwise stated. Data were checked for normality by Shapiro-Wilk test and analyzed by parametric tests when appropriate. Injection-pain scores rated on VAS were compared between the two injection protocols by Wilcoxon signed rank test and Friedman test of variance was used to compare each protocol across time. PPTs and pain area parameters following tonic pressure stimulation and contraction-induced muscle pain were analyzed by 3-way repeated measures ANOVA and, when significant, were followed by Bonferroni corrected post-hoc tests. Within-subject factors were time (7 sessions), injection type (distributed vs. bolus), and site (most proximal, middle, most distal, EDL, VA). AUCs of the time versus pain-area relation following tonic pressure stimulation were analyzed by 2-way ANOVA. Daily functional pain (Likert score), evoked overall pain intensity (NRS), and size of overall pain area following contraction-induced pain, were compared between the two injection protocols by Wilcoxon signed rank test and adjusted for multiple comparisons by Bonferroni correction. Friedman test of variance was used to compare each protocol across time. When significant, post-hoc pairwise comparisons were performed using Wilcoxon signed rank test and all P-values were adjusted with Bonferroni correction. The statistical analysis was performed using SPSS (IBM SPSS version 24) and significance level was accepted at $P \leq 0.05$.

RESULTS

Pain intensity during injections

The continuous VAS pain profile showed a similar time course for both the distributed injections and the single-site bolus NGF protocol (Fig. 2). There was no difference in mean VAS score between the two protocols in the periods during the injections procedure and after the injections

were completed (Wilcoxon: P>0.214). There was no difference in mean VAS score between the three periods of sampling time for the distributed NGF injections ($X^2(2)=8.3$, P>0.05). However, the mean VAS score immediately after the injection procedure was significantly higher than the period during the injections (Wilcoxon: P=0.001) and the post injection period (Wilcoxon: P=0.006) for the single-site bolus NGF protocol (Fig. 2). Area under the VAS-time curve (VAS-area) was higher after the distributed injections compared with the bolus injection (VAS score: 2.0 ± 0.1 cm·s vs. 1.8 cm·s ± 0.1; z= -2.87, P=0.004). No pain at rest was reported in the following session (3 hours after, day 1, 3, 7, 14, and 21) after the injection procedure of both NGF protocols.

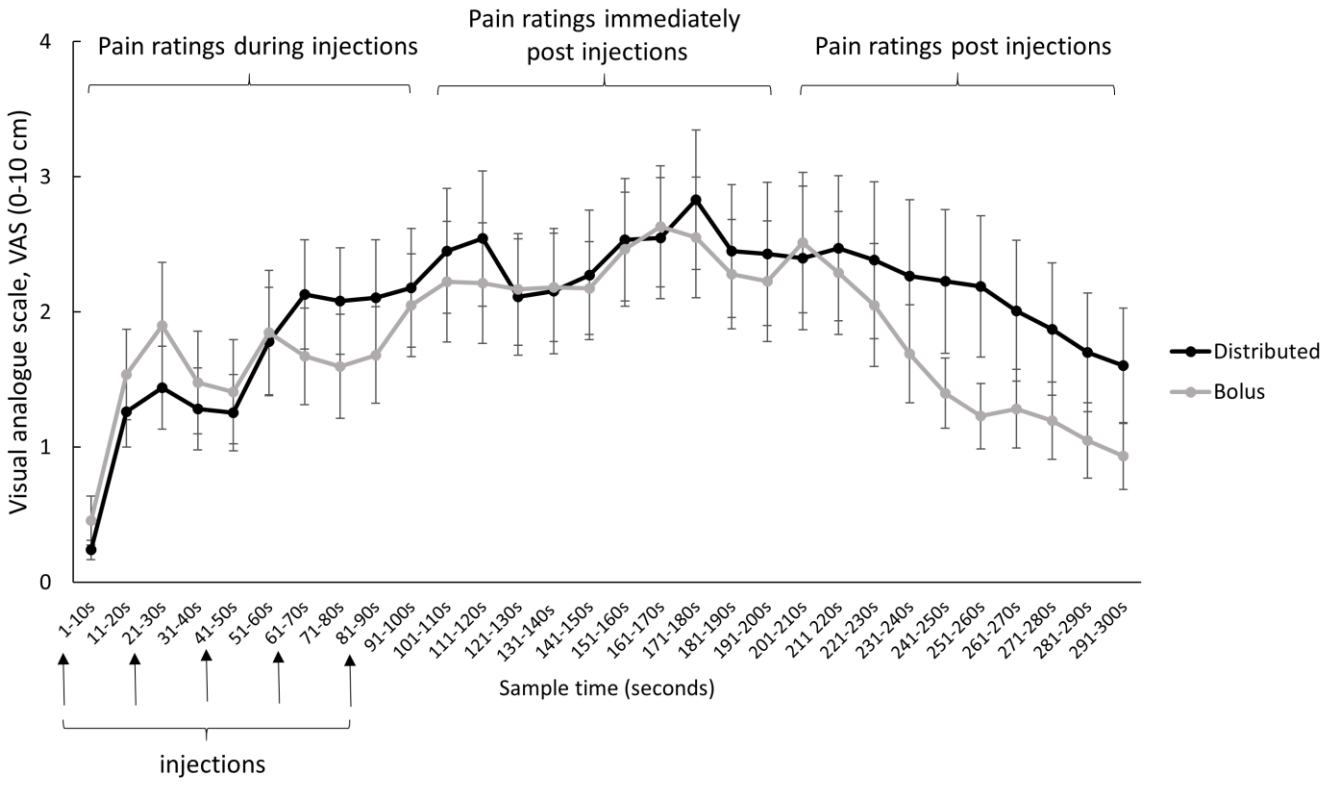

Fig. 2 Mean (± SEM, n=20) visual analogue scale (VAS) scores of pain intensity for the low-dose

distributed NGF injections (black line) and single-site bolus injection protocol (grey line). The individual injection was completed over approximately 10 s with a 10 s interval between injections as shown by the arrows. Original VAS scores were sampled with 1 Hz and are presented as averaged across 10 s intervals. Subjects rated their pain intensity continuously for 5 min, and the brackets indicate the periods during the injection procedure and after completion of the injections.

Pain diary

No data on the Likert pain scores were missing and no information was received on other issues such as deviated time point of the assessment. Compared with baseline, Likert scores of pain were higher following both the distributed injections ($X^2(5) = 89.9$, $P<0.005$, Wilcoxon: $P\leq0.005$) and the bolus injection protocol ($X^2(5) = 88.3$, $P<0.005$, Wilcoxon: $P\leq0.005$, Fig. 3) until Day12. There was no difference between the two injection protocols within each time point (average of 4 days, Wilcoxon: $P\geq0.06$). There was no difference in peak pain Likert scores (distributed: 3.0 ± 0.3, bolus: 2.9 ± 0.3; $z=-0.115$, $P=0.91$) or mean of the Likert scores over 21 days (distributed: 1.2 ± 0.6, bolus: 1.1 ± 0.5; $z=-1.800$, $P=0.072$) between the distributed and bolus injections. Additionally, no pain at rest was reported in the sessions (3 hours after, day 1, 3, 7, 14, and 21) after either NGF protocol.

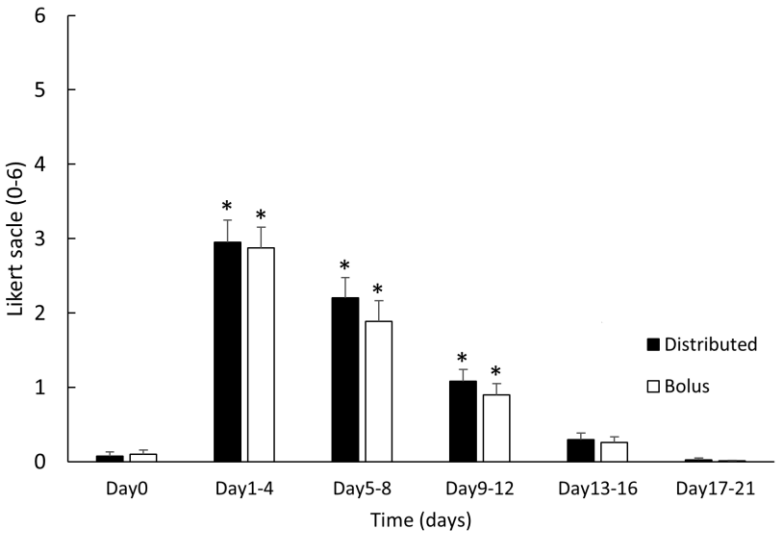

Fig. 3 Mean (± SEM, n=20) Likert scores from the pain diary for the distributed injections (solid bars) and single-site bolus injection protocols (open bars). Likert scores were averaged across 4 days. Significantly higher compared with baseline Day0 (*, P<0.005).

Pressure pain sensitivity

Results from ANOVA on PPT values, demonstrated a 3-way interaction between injection protocol, site, and time (ANOVA: F = 3.92, P<0.05). At the proximal injection site, PPTs were lower for the distributed and single-site bolus injection protocols at Day1, but higher at Day21 in comparison with baseline (Fig. 4A; post-hoc: P<0.05). Compared with baseline, PPTs at the middle injection site were reduced after 3 hours, at Day1 and Day3, and increased at Day21 following the distributed

injections (Fig. 4B; post-hoc: $P<0.05$). After the single-site bolus injection, PPTs at the middle injection site were reduced at Day1, Day3, and increased at Day21 compared with baseline (post-hoc: $P<0.05$). Compared with baseline, PPTs at the distal injection site were reduced at Day1, Day3, and increased at Day21 following the distributed injections (Fig. 4C, post-hoc: $P<0.05$). After the single-site bolus injection, the PPTs at the distal injection site were reduced after 3 hours, at Day1, but increased at Day14 and Day21 compared with the baseline (post-hoc: $P<0.05$). At the EDL muscle, PPTs were reduced after 3 hours and at Day1 when compared with the baseline after the single-site bolus injection (Fig. 4D, post-hoc: $P<0.05$).

Compared with the single-site bolus injection, PPTs at Day1 were reduced following the distributed injections at the most distal site (post-hoc: $P=0.021$), but increased at the EDL muscle (post-hoc: $P=0.013$). At Day3, compared with the bolus injection, PPTs were reduced after the distributed injections at the proximal site (post-hoc: $P=0.036$) and the distal site (post-hoc: $P=0.002$).

Pain areas following tonic pressure stimulations were not significantly affected across injection protocols or time (results are presented in supplementary material, Fig. S1-S4).

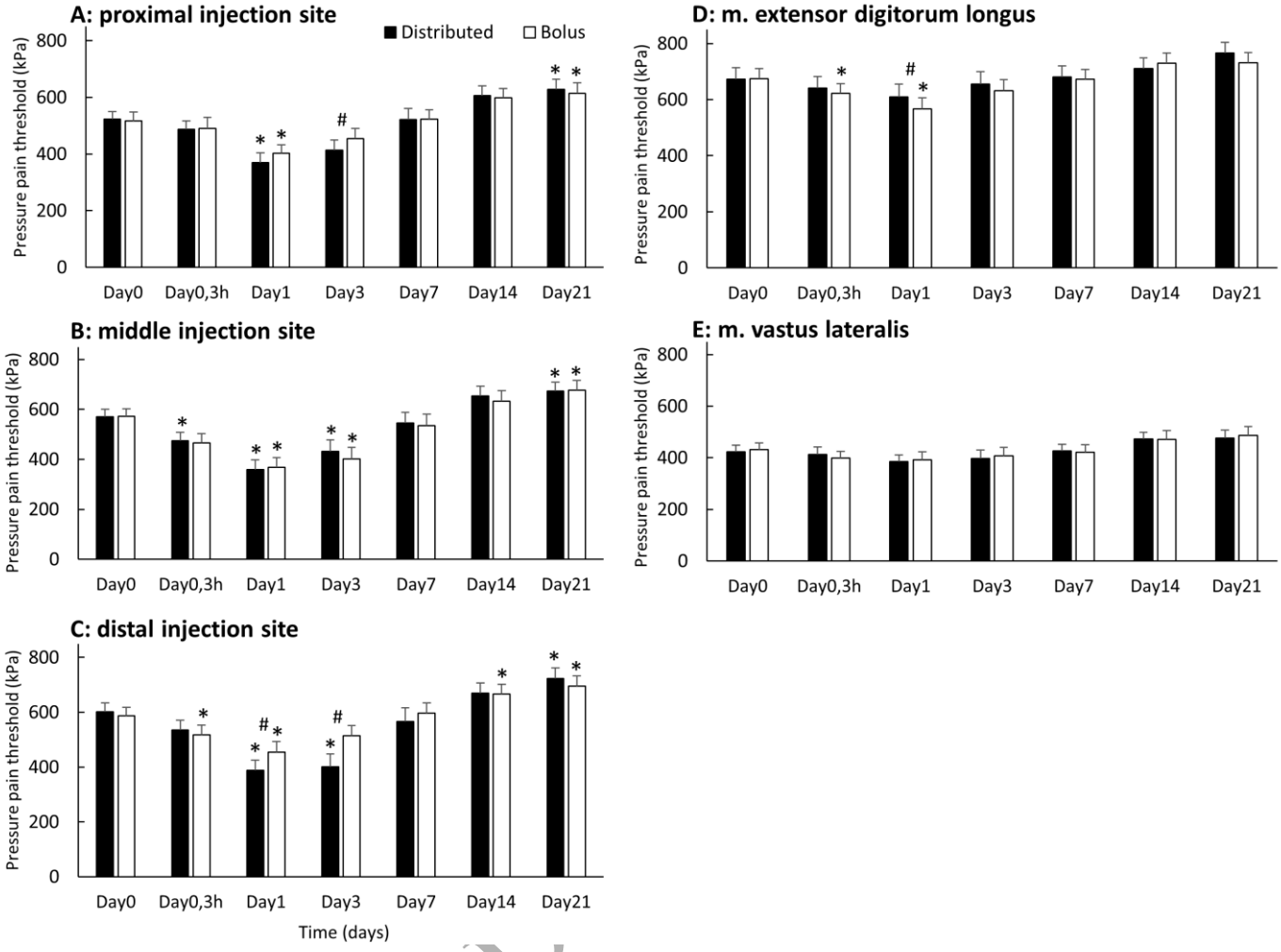

Fig. 4 Mean (± SEM, n=20) pressure pain thresholds (PPTs) for the distributed NGF injections (solid bars) and single-site bolus NGF injection (open bars) at each assessment site: **A)** proximal injection site, **B)** middle injection site, **C)** distal injection site, **D)** m. extensor digitorum longus/EDL, **E)** m. vastus lateralis/VL. PPTs were recorded at baseline (Day0), and 3 hours (Day0), Day1, Day3, Day7, Day14, and Day21 after injections. Significantly different compared to baseline Day0 (*, P<0.005) or compared to the single-site bolus NGF injection within the same day (#, P<0.01).

Contraction-induced muscle pain

Following both injection protocols, larger overall pain areas were found after the contractions of the TA muscle after 3 hours, at Day1, Day3, and Day7 when compared with baseline (Fig 5, Fig. 5A: Distributed: $X^2(6) = 79.73$, P<0.005; Wilcoxon: P<0.005. Bolus: $X^2(6) = 78.28$, P<0.005; Wilcoxon:

P<0.005).

The area under the curve (AUC), relating pain area and time, was higher for the distributed injections compared to the single-site bolus injection (2503425 ± 416928 arbitrary units vs. 1014690 ± 173412 arbitrary units, t = 4.446, P<0.05).

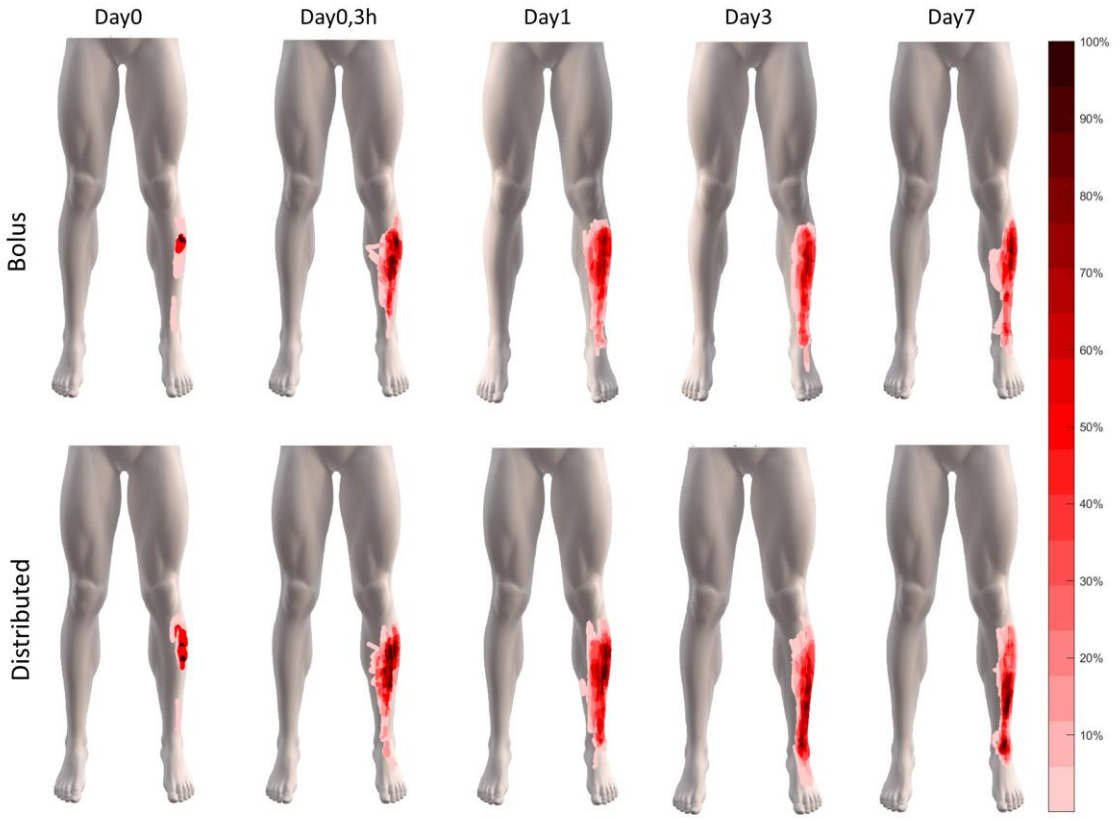

Fig. 5 Superimposed pain drawings from all 20 participants following the contraction task at baseline (Day0), 3 hours after (Day0,3h), Day1, Day3, and Day7. Before image processing, all overlays were mirrored to the same side for visual comparison between the two injection protocols over time. Darker regions of the overlay represents a higher frequency of overlapping pain drawings.

Following the contractions of the TA muscle, the pain area length (distal to proximal) was increased at 3 hours and at Day1, Day3, and Day7 after both injections protocols when compared with baseline (Fig. 5, Fig. 6B; ANOVA: $F=29.0$, $P<0.05$, post-hoc: $P<0.05$). The width (medial to lateral) of the pain area was increased at 3 hours and at Day1, Day3, and Day7 after both injections protocols when compared with baseline (Fig. 5, Fig. 6C; ANOVA: $F=41.7$, $P<0.05$, post-hoc: $P<0.05$).

The pain NRS scores reported after the contractions of the TA muscle were higher after 3 hours, at Day1, Day3 and Day7 when compared to the baseline after the distributed injections ($X^2(7) = 116.05$, $P< 0.005$; Wilcoxon: $P<0.005$) and single-site bolus injection ($X^2(7)=104,422$, $P<0.005$; Wilcoxon: $P<0.005$). Comparing the two injection protocols, higher NRS pain scores were found at Day-3 in the leg receiving the distributed injection (Fig. 6D, Wilcoxon: $z = -3,181$, $P<0.005$).

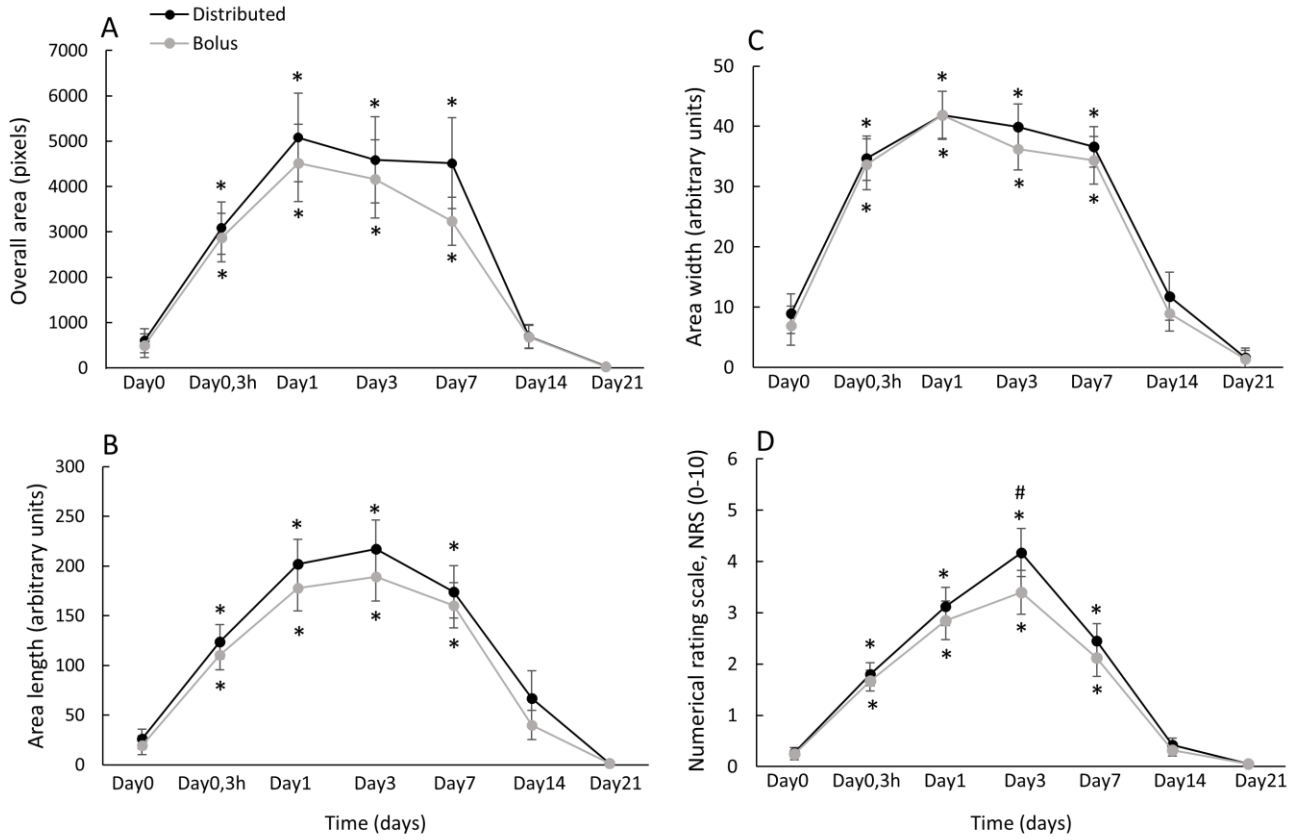

Fig. 6 A) Mean (± SEM, n=20) overall pain area, **B)** area length, **C)** area width, and **D)** pain intensity (NRS), following the contraction task for the distributed injections (black) and single-site bolus injection (gray) protocols. Area parameters and NRS were reported at baseline (Day0), 3 hours (Day0) after and at Day1, Day3, Day7, Day14, and Day21. Significantly different compared to baseline (*, P<0.05). Significant difference between the two injections protocols (#, P=0.001).

DISCUSSION

The novel findings of this paper are that sensitization to pressure and muscle contraction were found over a larger area of the muscle when the same NGF dose was distributed at various

injection sites, as compared to a single NGF bolus. In line with previous studies, spontaneous pain at rest was absent in both injection protocols. Despite that, weak pain was observed during both NGF protocols, and higher pain intensity favored the distributed NGF injections.

NGF-induced injection pain

In previous studies, a single injection of NGF (5µg), as well as control injection of isotonic-saline, into the masseter, tibialis anterior or trapezius muscles induced almost no pain that was reported after the injection was completed [1,19,29]. Andersen et al. 2018 [1] described a low immediate pain after NGF injection in the TA muscle (VAS score: 0.5±0.3/10 cm), whereas in this study, an immediate weak pain (VAS scores: 2.0±0.1 cm vs 1.8±0.1 cm/10 cm) was reported for both the distributed and single-site protocols. Contrary to prior studies, these VAS pain intensity scores were assessed during the injections, which may explain the higher pain intensities. The post-injection pain ratings may however reflect NGF-induced pain intensity in a more accurate way, as the pain rating would better reflect the NGF substance and not the injection procedure. Following a daily NGF (5µg) injection protocol, repeated over 3 consecutive days, Hayashi et al. [12] reported a low intensity pain immediately after the 1st injection and a significantly higher pain intensity after the 2nd and 3rd injection of NGF, respectively. The subsequent higher pain intensities are likely the result of an already sensitized TA muscle. Facilitated pain after daily NGF injections into the same tissue is in line with animal findings, where subthreshold potentials produced in rat dorsal horn following one NGF injection facilitated more action potentials after additional NGF injections [15]. Despite the weak pain associated with the NGF injections in the present study, it is unclear whether the excitation [14] and nociceptor discharge [30] would be similar to what is observed in animal findings. Interestingly, pain intensity increased on subsequent injection in both protocols with a slower decrease following NGF injection in the distributed protocol compared to the single NGF protocol. However, as this study did not include a positive control-injection protocol, the contribution of the injection procedure to the immediate pain report cannot be disentangled.

Spontaneous pain and muscle pain during daily function

As a mild to moderate pain is associated with intravenous administration of NGF in clinical testing [3,21], it was speculated that a single-site bolus injection of NGF (5µg) deposited into the muscle would saturate the tissue at injection-site and excite all available nociceptors albeit not sufficient to evoke spontaneous pain. Hence, distributing the NGF dose over a larger area of the muscle could potentially lead to a spontaneous pain response at rest. However, no pain at rest was reported in the present study after either injection protocol in the days post-injection. Only one previous study reported a low-intensity pain at rest bilaterally over the supraspinatus muscles 1 day post NGF injection [8]. However, whether this finding stands out as a single case has not previously been clarified. Additionally, pain responses to NGF injection in tissues with a dense innervation of nociceptors such as the skin [7,24] and muscle fascia [5] have revealed inconsistent findings. Dyck et al. [7] observed severe myalgia in a subject lasting up to 2 days following intradermal NGF injection whereas Deising et al. [5] found no acute pain after NGF injection into the fascia. In the patella fat pad, NGF induced a moderate-severe knee pain that was experienced in few subjects with pain lasting up to 1-3 months [18]. It is unclear though if NGF caused the ongoing pain or if it resulted from an underlying pathological condition present prior to participation in these studies [18].

Self-perceived muscle pain during daily function as assessed by a Likert scale is commonly associated with intramuscular NGF injection [1,4,15,16]. In the present study, a peak pain Likert score was present around Day1 to Day4 for both NGF injection protocols (3.0±0.3 and 2.9±0.3) declining over the subsequent 12 days. A previous study using 5µg NGF showed a peak in Likert pain score at Day1 (2.0±0.2), only lasting up to Day7 [1]. As the same dose of 5µg NGF was used in the single-site bolus NGF protocol in this study, similar findings could have been expected as described in the previous NGF study. However, since this was not the case, it could be speculated that the higher peak pain intensity and longer duration of self-perceived muscle pain associated with both injection protocols was mainly driven by perception of the distributed NGF injections.

NGF-induced hyperalgesia

The distributed and the single-site injection protocols provoked muscle hyperalgesia after 3 hours

that continued up to Day3 before returning to baseline values on Day7. The onset and time course of hyperalgesia are consistent with previous injection studies of NGF (5µg) into the TA [1,12,18]. Additionally, increased PPTs were seen after 14-21 days following both injection protocols. The increase in PPTs observed after a period of muscle hypersensitivity has been shown in other long-term studies with repeated pressure stimulation, both with and without prior NGF injection [1,16,29], and may possibly reflect familiarization to the test procedure, although the mechanism is still unclear.

Comparing the individual injection sites between the two protocols, no difference in sensitization was seen at the middle injection (site 3) receiving either 1 or 5µg NGF. Despite that the total dose of NGF was the same in both protocols, this finding suggests that 1 µg NGF is equally adequate for sensitizing the receptors at the site of injection. Likewise, the PPTs at the most proximal (site 1) and distal (site 5) sites were decreased at Day1 and Day3 following the low-dose distributed NGF injections.

Although site 1 and site 5 were injected with saline in the single-site bolus protocol, PPTs were decreased at Day1. A previous study found spreading muscle hyperalgesia from NGF injection-site (5µg) after 1 day lasting up to Day4 [1]. Decreased PPTs at proximal and distal sites in the single-bolus protocol could possibly be driven by a short-lasting spreading effect from the middle NGF injection site although only present at Day1. As all injection sites included NGF in the distributed injections, it is unclear if a spreading effect of NGF based on a central mechanism was present between sites in this protocol. In addition, there was no extension of local pain areas assessed in the days after tonic pressure stimulation in either protocol. In contrast, Hayashi et al. [12] showed enlargement of pressure-induced pain area following tonic pressure stimulation, that developed across the time course of daily repeated NGF injections. In the present study, the 120% pressure stimulation was normalized to the reduced PPTs as opposite to Hayashi et al. that applied a pressure equal to 120% of baseline values. Tonic pressure stimulation has not previously been assessed in studies using a single NGF injection. However, when compared with the present findings, the effect presented by Hayashi et al. may be due to the daily injections or the relatively

higher-pressure stimulation given post NGF injections. At the EDL, decreased PPTs were seen after the single-site NGF injection 3 hours after and at Day-1. As this muscle shares the same neural innervation as the TA, this site was chosen to investigate widespread effects of NGF. Andersen et al [1] showed an increase in muscle pain sensitivity at the EDL site when NGF was injected into the TA, although not significantly different from baseline. However, the web space between 1st and 2nd metatarsal was more sensitive to pressure pain stimulation following NGF at Day1 [1]. There was no change in muscle sensitivity at the VL muscle, suggesting that NGF was not able to cause any effects extra-segmentally.

Contraction-induced muscle pain

Increased pain ratings and enlargement of pain areas were found after the contraction task in both protocols. Consistent with other studies, increased pain intensity during muscle contraction was reported in the muscle receiving NGF compared to a control injection in the leg [1], shoulder [8,19] and arm [4]. Normally, a contraction of the muscle is not painful and additionally, contraction evoked pain is not evident in other injection-based pain models [31]. Compared with the single-site bolus NGF injection, higher pain intensity was reported after the distributed NGF injections, and likely result from the activation of sensitized nociceptors throughout the entire muscle compartment. Such finding may reflect spatial summation of nociception, evoked by stimuli over a wide distance, and presumably across spinal segments [28]. In support of this, the involvement of spatial summation in muscle pain was demonstrated following injections of hypertonic saline given a spatially separated sites in the TA muscle compared with a bolus injection [10]. As facilitated summation of pain is likely implicated in clinical pain conditions, reflecting such feature is clearly favored by a distributed injection procedure and should be studied further in future NGF pain models.

Conclusion

This study showed that spatially distributed low-dose injections of NGF along the TA muscle induced pronounced muscle hyperalgesia, functional muscle pain, and relatively larger contraction-induced areas of pain, although not evoking spontaneous pain, in the following days.

Low doses of NGF adequately sensitize muscle nociceptors locally and distributed low doses of NGF sensitize a larger volume of muscle tissue as compared to a single-site NGF bolus injection. The spatially distributed NGF-injection protocol is a more efficient use of NGF and may better mimic aspects of clinical muscle pain as a larger proportion of the muscle compartment is affected. Such refined NGF pain models offer advantages for future studies of prolonged muscle pain and hyperalgesia.

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FIGURE LEGENDS

Figure 1. A) Illustration of the five injection sites (1, 2, 3, 4, 5) within the tibialis anterior muscles (TA) for each injection protocol, and assessment sites for mechanical pressure stimulation

(proximal injection site, middle injection site, distal injection site, m. extensor digitorum longus/EDL, m. vastus lateralis/VL). **B)** Experimental timeline of the seven assessment sessions and the assessment protocol.

Figure 2. Mean (± SEM, n=20) visual analogue scale (VAS) scores of pain intensity for the low-dose distributed NGF injections (black line) and single-site bolus injection protocol (grey line). The individual injection was completed over approximately 10 s with a 10 s interval between injections as shown by the arrows. Original VAS scores were sampled with 1 Hz are presented are averaged across 10 s intervals. Subjects rated their pain intensity continuously for 5 min, and the brackets indicate the periods during the injection procedure and after completion of the injections.

Figure 3. Mean (± SEM, n=20) Likert scores from the pain diary for the distributed injections (solid bars) and single-site bolus injection protocols (open bars). Likert scores were averaged across 4 days. Significantly higher compared with baseline Day0 (*, P<0.005).

Figure 4. Mean (± SEM, n=20) pressure pain thresholds (PPTs) for the distributed NGF injections (solid bars) and single-site bolus NGF injection (open bars) at each assessment site: **A)** proximal injection site, **B)** middle injection site, **C)** distal injection site, **D)** m. extensor digitorum longus/EDL, **E)** m. vastus lateralis/VL. PPTs were recorded at baseline (Day0), and 3 hours (Day0), Day1, Day3, Day7, Day14, and Day21 after injections. Significantly different compared to baseline Day0 (*, P<0.005) or compared to the single-site bolus NGF injection within the same day (#, P<0.01).

Figure 5. Superimposed pain drawings from all 20 participants following the contraction task at baseline (Day0), 3 hours after, Day1, Day3, and Day7. Before image processing, all overlays were mirrored to the same side for visual comparison between the two injection protocols over time. Darker regions of the overlay represents a higher frequency of overlapping pain drawings.

Figure 6. A) Mean (± SEM, n=20) overall pain area, **B)** area length, **C)** area width, and **D)** pain intensity (NRS), following the contraction task for the distributed injections (black) and single-site bolus injection (gray) protocols. Area parameters and NRS were reported at baseline (Day0), 3 hours (Day0) after and at Day1, Day3, Day7, Day14, and Day21. Significantly different compared to

baseline (*, P<0.05). Significant difference between the two injections protocols (#, P=0.001).