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BY
LINE BAY SØRENSEN

DISSERTATION SUBMITTED 2020

AALBORG UNIVERSITY
DENMARK

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PHD THESIS

by

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Dissertation submitted

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Line obtained a B.Sc. in Medicine with Industrial Specialization and M.Sc. in Translational Medicine from the Faculty of Medicine at Aalborg University, Denmark in 2016. Hereafter she enrolled as a Ph.D. fellow, working at the Center for Neuroplasticity and Pain (CNAP) under the supervision of Professor Thomas Graven-Nielsen and co-supervision of Associate Professor Parisa Gazerani. Her main research focus has been on human long-term pain models by application of the neurotrophic protein, Nerve Growth Factor (NGF) injected into muscle. In this respect, different NGF injection protocols have been explored, and subsequent responses on pain perception and muscle sensitivity have been investigated and assessed with methods testing the effect of NGF on both peripheral and central mechanisms.

ENGLISH SUMMARY

Chronic muscle pain affects a large proportion of the adult population. In patients with muscle pain, increased sensitivity of deep tissue structures is a major complaint that over time becomes more painful and interferes with daily activities. Both peripheral and centrally mediated pain mechanisms have been suggested to play important roles in generating altered pain processing, although their contribution to maintenance of long-term symptoms such as muscle hyperalgesia and evoked pain is less clear. One of the greatest challenges in treating these conditions is the present lack of successful pain management, possibly due to an inadequate understanding of muscle pain pathology and the involved mechanisms in pain signaling pathways.

Pain research has advanced through the development and use of various translational models in which pain can be evoked in a standardized and reproducible manner. Different methods are often utilized to induce experimental muscle pain. However, few can produce long-lasting symptoms that mimic clinical pain characteristics. Evidence from clinical and experimental studies suggest that the neurotrophic factor, Nerve Growth Factor (NGF) is a key mediator of nociception and is involved in various pain states. Studies have examined NGF's sensitizing effects on both peripheral and central mechanisms and demonstrated profound hyperalgesic effects after NGF administration in tissues. Pain responses in NGF models are still controversial, and the mechanisms affecting NGF-induced muscle pain sensitivity are poorly understood and have not been clearly demonstrated in humans. Therefore, the aims of this Ph.D.-project were to explore evoked and non-stimulus evoked (spontaneous) pain responses, and the effects of NGF administration on pain sensitivity using methods assessing both peripheral and central mechanisms, in NGF pain models investigated in healthy humans.

The first study showed that compared with a single-site bolus injection of NGF of the same total dose, five injections of low-dose NGF spatially distributed into the tibialis anterior muscle induced pronounced muscle hyperalgesia, activity-evoked pain and increased contraction-evoked pain with larger pain areas. The second study demonstrated that acute acidification of the muscle environment (i.e. ischemic muscle contractions) after NGF injections did not facilitate muscle pain sensitivity, but NGF may sensitize muscle nociceptors possibly through the responsiveness of chemo-sensitive channels as higher contraction pain was evoked with the NGF injected muscle during ischemia as compared to a non-sensitized muscle. In the third study, subjects sensitized by three low-dose NGF injections separated by 2-day intervals induced prolonged activity-evoked pain, but with less severe intensity. Additionally, ischemic contraction-evoked pain was increased with prolonged NGF sensitization. However, maintained NGF-induced sensitization did not significantly affect central mechanisms, as assessed by temporal summation of pain and conditioned pain modulation.

Taken together, the current Ph.D. studies have clarified significant details of the NGF pain model, which is an important step forward that offers advantages for future studies of prolonged muscle pain and muscle hyperalgesia. The current findings illustrate that NGF plays a role in sensitizing peripheral afferents over a large area of the muscle, producing more widespread pain areas and altered responses during ischemic conditions. Hence, these newly-developed NGF models may better mimic some aspects of clinical muscle pain and peripheral muscle sensitization with ischemic complicity.

DANSK RESUME

Kroniske muskelsmerter påvirker en stor del af den voksne befolkning. En øget smerteoverfølsomhed af de dybe vævsstrukturer er ofte anledning til stor bekymring hos patienter med muskelsmerter, da smerten kan forværres og have en negativ indvirkning på dagligdagens gøremål og aktiviteter. Det er blevet foreslået, at både perifere og central-medierede smertemekanismer i centralnervesystemet ligger til grund for ændret smerte signaliering i smertesystemet, men uvist er disse mekanismers medvirken i vedligeholdelsen af de længerevarende symptomer i form af muskel hyperalgesi og fremkaldt smerte. En af de største udfordringer i behandlingen af disse symptomer er den nuværende mangel på vellykket smertehåndtering, der sandsynligvis skyldes en stadig mangelfuld forståelse af muskelsmertens patologi og de involverede mekanismer i smertesystemet.

Gennem tiden har smerteforskningen udviklet sig via brugen og udviklingen af forskellige translationelle smertemodeller, hvorved smerte kan fremkaldes på en standardiseret og reproducerbar måde. Forskellige metoder kan bruges til at frembringe en eksperimentel smerte. Dog er det kun et fåtal af disse metoder, der kan frembringe de længerevarende smertesymptomer og som efterligner det smertebillede, man ser hos patienter med kroniske muskelsmerter. Viden fra kliniske og eksperimentelle studier påpeger, at det neurotropiske protein, Nerve Growth Factor (NGF) er en hovedaktor i nociception og desuden, involveret i flere forskellige smertetilstande. Flere studier har undersøgt NGF og den effekt som proteinet udøver på både perifere og central-medierede mekanismer i centralnervesystemet som ses i form af en dybtgående smerteoverfølsomhed (hyperalgesisk påvirkning) af forskellige vævstyper. Smerteresponset fremkaldt i NGF-modellerne er dog stadig kontroversiel og mekanismerne, der kan have indvirkning på den muskulære smerteoverfølsomhed, der ses efter administreringen af NGF er stadig uvis og ikke nok undersøgt i humane modeller. Derfor har dette Ph.d.-projekt haft til formål at undersøge både fremkaldt og spontan NGF-induceret smerte, og effekten af administrationen af NGF på muskulær smerteoverfølsomhed ved brug af metoder, der undersøger både perifere og central-medieret smertemekanismer, fremkaldt i raske og humane NGF-induceret smertemodeller.

Det første studie sammenlignede en enkelt bolus injektion af NGF (samme totale dosis) med fem lav-dosis NGF injektioner distribueret over tibialis anterior musklen og viste, at denne sidste injektions metode fremkaldte en dybtgående muskulær smerteoverfølsomhed, aktivitets-provokeret smerte og en øget kontraktionssmerte med et større fremkaldt smerteområde over musklen. Det andet studie viste, at akut iskæmisk påvirkning af muskelmiljøet (iskæmiske muskel kontraktioner) efter injektion af NGF ikke fremkaldte en muskulær smerteoverfølsomhed. Men det kan tænkes, at NGF muligvis påvirker muskel nociceptorerne via indvirkningen fra kemo-sensitive receptorer, da en højere kontraktionssmerte var fremkaldt i musklen under

iskæmi efter at NGF var administreret sammenlignet med en ikke-sensibiliseret muskel. Det tredje studie viste, at hos de personer, der udviste en muskulær smerteoverfølsomhed efter tre gentagne lav-dosis NGF injektioner givet hver anden dag, blev fremkaldt en længerevarende, men mindre intens, aktivitets-provokeret smerte. Derudover viste studiet, at iskæmisk kontraktionssmerte var øget under en længerevarende påvirkning af NGF, der dog ikke havde nogen signifikant indvirkning på de central-medieret mekanismer, målt ud fra ændringer i opfattelsen af faciliteret smertepåvirkning (temporal summation of pain) og under smertemodulering (conditioned pain modulation).

Samlet set har dette Ph.d.-projekt afdækket noget signifikante og nye detaljer af NGF smertemodellen, som kan have væsentligt indflydelse på fremtidlige studier, der har til hensigt at undersøge længerevarende muskelsmerter og muskulær smerteoverfølsomhed. Ydermere, resultaterne fra disse studier viser, at den NGF-induceret sensibiliseringen af perifere afferente nervefibre over et større område af musklen fremkalder et større smerteområde og et ændret smerterespons under påvirkningen af iskæmiske muskelkontraktioner. Disse nye detaljer kan om muligt være med til, at NGF-modellen nu også kan efterligne kliniske aspekter af muskelsmerte og muskeloverfølsomhed med involveringen af perifere smertemekanismer og tilstand af iskæmi.

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PREFACE

The present Ph.D. thesis summarizes the work that was performed between August 2016 and December 2019 at The Center for Neuroplasticity and Pain (CNAP), SMI®, Department of Health Science and Technology, Aalborg University, Denmark. The work has been fully supported by the Danish National Research Foundation (DNRF121).

The aims of this thesis were to explore new aspects of the NGF human pain model by assessing evoked and non-stimulus evoked pain responses to novel i.m NGF injection protocols, as well as investigating the effects of NGF-induced sensitization using methods assessing both peripheral and central mechanisms.

The thesis is organized as an extended summary that will provide a brief overview of the research topic, review the original papers, and highlight the most important findings achieved from this work. As such, the first chapter will introduce NGF mechanisms and NGF-induced manifestations of muscle sensitization in human pain models in light of previous work, eventually leading up to the objectives and aims of this thesis that are presented at the end of the first chapter. The second and third chapters cover the methodology used in this study to induce experimental muscle pain by use of novel intramuscular NGF injection protocols, followed by assessing pain responses and NGF-induced sensitization in healthy humans. Additionally, the main findings are discussed across studies at the end of chapter 2 and chapter 3, respectively, in relation to the pain and sensitization induced by the different NGF protocols. The fourth chapter presents the methods used to assess changes related to central pain mechanisms followed by discussing the main findings on the effect of central effects of NGF-induced pain and muscle sensitivity at the end of this chapter. Finally, the fifth chapter outlines a brief conclusion on the studies comprising this thesis, and presents future implications for the novel NGF injection protocols investigating prolonged pain and muscle hyperalgesia

The content of this PhD thesis is based on three original papers, with two published in international peer-reviewed journals, and the third one under peer review.

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CHAPTER 1. INTRODUCTION

Chronic muscle pain is a major health problem affecting a large proportion of the adult population (1,2). Adequate understanding of the pathophysiology and underlying mechanisms are lacking, and thus improving understanding of muscle pain pathology and involved pathways is essential for optimizing treatment of affected individuals (3). In clinical settings, deep tissue pain, including muscle pain, is often difficult to characterize because it often involves local as well as widespread pain areas (4). Additionally, the experience of muscle pain may not only persist but is often described as tender and sore muscles that become more painful over time with daily activities (i.e. muscle hyperalgesia). Both peripheral and centrally mediated mechanisms are suggested to play important roles in muscle pain development, although less clear is their contribution to the maintenance of long-term symptoms such as muscle hyperalgesia and evoked pain (5). From a clinical perspective, deep tissue pain is poorly understood compared with skin pain (6), and therefore highly relevant for further investigation.

1.1. HUMAN PAIN MODELS

Pain research has advanced through the development and use of various translational human pain models in which pain can be evoked in a standardized and reproducible manner, and be measured or assessed with standardized tools or techniques (7). Based on such models, specific pain mechanisms can be studied and later applied to fill gaps in our knowledge. Characterizing different aspects of pain symptoms evoked experimentally in healthy humans, can lead to further insights in some of the underlying mechanisms, and yield new methods to study pain development and maintenance.

Current human pain models entail both exogenous and endogenous methods to induce and assess muscle pain and pain sensitivity (8,9). Endogenous models can be used to reflect pathological muscle pain by use of natural stimuli (e.g. ischemia and exercise) (8). Exogenous techniques include different modalities such as electrical, mechanical, and chemical stimulations (9), which are applied based on the mechanisms being studied. For instance, a variety of algogenic substances such as bradykinin, glutamate, serotonin, histamine, and prostaglandin E_2 have been injected into muscles and assessed for their ability to evoke pain and muscle hyperalgesia (10,11). Moreover, several experimental pain models have been developed using intramuscular (i.m.) injection of hypertonic saline, capsaicin, and acidic buffers, which induce a chemical pain that closely resembles the manifestations of the pathological pain with localized and spread pain areas (12,13). This in turn, has helped in understanding how short-lasting pain (minutes) affects e.g. pain intensity, pain quality, and motor performance (14). However, these models typically work over a short time duration and could only investigate acute pain development.

1.2. HUMAN NGF PAIN MODELS

Over the past decades, long lasting pain models have been developed using i.m application of the neurotrophic protein, Nerve Growth Factor (NGF). Normally, NGF promotes survival and growth of the nervous system (15); however, NGF is also an important mediator of persistent pain processing (16,17) and modulates several gene processes following nerve injuries and inflammation when expression of NGF is increased subsequently (18). However, the exact mechanisms by which NGF functions in the pain system are sparsely understood, and both peripheral sensitization and an altered central processing are suggested to play a role. Indeed, maintained nociceptor activity is suggested to occur through the process of retrograde transport of NGF to the dorsal root ganglia (DRGs) (19,20) by peripheral nerve endings, followed by increasing gene expression in central nociceptor terminals (17). A prior human study (21) showed that repeated injections of NGF (5µg) induced prolonged muscle pain (10-days) and muscle hyperalgesia up to 3-days with affected muscle pain sensitivity outside the hyperalgesic area, increased pressure-induced pain areas, and facilitated temporal summation of pain. Since no NGF dose-response relationship has been established, it is unknown whether repeated injections of a lower dose NGF (1µg) induce and maintain muscle hyperalgesia in a similar manner. In the prior NGF study (21), the findings suggest that the mechanisms of NGF-induce pain and muscle hyperalgesia may not only be based on local peripheral sensitization, but also may involve changes of central mechanisms over time.

NGF is also considered an algogenic substance mediating key functions of inflammatory hypersensitivity (17). Following experimental or pathological inflammation, endogenous NGF levels are substantially increased in peripheral inflamed tissues, as NGF production and secretion from other cell types are strongly mediated by inflammatory cytokines (22). Hence, NGF becomes a key driver of peripheral sensitization (23). Tissue acidosis is an accompanying feature of inflammation and contributes to symptoms such as pain and hyperalgesia (24). In animals, injection of various acidic solutions activates chemo-sensitive channels such as acid-sensing ion channels (ASICs) and the transient receptor potential vanilloid 1 (TRPV1), with observed mechanical hyperalgesia following the subsequent stimulation (25,26). Moreover, NGF has shown to influence expression of ASICs genes that parallel an increased ASIC-like mediated current in rat DRGs (27,28) and also NGF modulates TRPV1 activity (29). In a current human study, NGF-induced mechanical hyperalgesia assessed at the tibialis anterior (TA) muscle was further facilitated by a subsequent acidic infusion 24 hours after injection of NGF (30). The exact mechanisms behind NGF-induced sensitization under acidic stimulation are not fully known; however, lowering the level of pH might trigger the opening of local TRPV1 and ASICs (31,32), and if sensitivity of TRPV1 and ASICs are facilitated by NGF, this might explain NGF-induced acidic-induced pain by NGF-induced facilitation.

Acidification of the muscle milieu can also be induced by ischemic muscle contractions, in which the muscle receives an insufficient amount of oxygen for its metabolic need and the level of pH decreases (32). In fact, work by Issberner et al. (33) showed that the intracellular level of pH in the forearm muscle decreased from 7.4 to 7.0 during the submaximal effort applied in a tourniquet technique. In this study, a modified version of the tourniquet technique was applied by use of a pressure cuff mounted over the thigh, which was inflated to occlude blood flow in the working TA muscle, and induced an acute ischemic condition. Additionally, NGF is involved in generating and facilitating evoked pain following daily activities (34–36) and muscle contractions (37). Moreover, a moderate and intense ischemic pain is evoked if the occluded limb is exercising (38). However, it is unknown whether ischemic-contraction evoked pain would be facilitated during NGF peak-sensitization and with maintained NGF-induced muscle hyperalgesia e.g. with repeated NGF injections, and therefore, this was a subject to explore in this project.

Elevated levels of endogenous NGF have been measured in the cerebrospinal fluid of patients with chronic headache and fibromyalgia, where pain is a prominent symptom (39), and moreover, detectable NGF levels have been found in patients associated with various painful inflammatory pain condition (40,41). Early clinical trials evaluating the therapeutic potential of NGF found that the dose-limiting effect of NGF (≥1μg/kg) induced muscle myalgia in both healthy volunteers (42) and patients with sensory neuropathies (43). Additionally, the lack of acute pain during NGF i.m application in human pain models seems to be a puzzling issue that remains open for further investigation. Although, NGF has shown prominent excitatory action on rat high threshold mechanical afferents (group IV) (44), no immediate pain has been reported during i.m single injection of NGF in previous human studies, or reported as spontaneous pain (i.e. non-stimulus evoked pain) in the days post-injection (34,36,37). This lack of pain after NGF injection also contrasts the immediate and acute pain that normally appears when other exogenous injection-based methods are applied (11). To elucidate if the lack of pain was a dose-related issue, a single high dose NGF (15μg) was injected into the TA muscle (Graven-Nielsen, unpublished data), albeit no acute pain was reported during or immediately after NGF injection. Hence, application of smaller dosages of NGF may be an important aspect to study next in human NGF pain models. As muscle pain was observed after systemic intravenous (i.v) administration in the early clinical testing (42), it could be speculated that NGF would reach the muscle nociceptors in a distributed manner and at a lower concentration (e.g. due to spatial summation of nociception), albeit enough to elicit pain. This was the base for the injection method explored in Study 1 using a low-dose spatially distributed NGF i.m injection protocol. Furthermore, such injection protocol affects a larger proportion of the TA muscle compartment, and was additionally used in Study II, exploring effects of NGF-sensitization and ischemic-contraction evoked pain. Lastly, effects of low-dose repeated NGF injections were explored in Study III.

1.3. AIMS OF THE PHD PROJECT

A number of human studies have examined NGF's sensitizing effects on both peripheral and central mechanisms and demonstrated profound muscle hyperalgesia lasting for days after singe i.m injection of NGF (*Appendix A*). Changes of central mechanisms during prolonged NGF-sensitization are only partly demonstrated and pain responses in NGF models are still controversial, though NGF seems to play a prominent role in the generation and facilitation of evoked pain and muscle sensitization. The mechanisms underlying NGF-induced muscle pain are still poorly understood and have not been adequately investigated in humans.

Therefore, the overall aims of this Ph.D.-project were to explore new aspects of the NGF human pain model assessing evoked and non-stimulus evoked (spontaneous) muscle pain responses to novel i.m NGF injection protocols, as well as investigating the effects of NGF-induced sensitization, using methods to assess both peripheral and central mechanisms in healthy humans. A schematic overview of the dissertation and included studies can be seen in (Fig. 1.1).

1.4. HYPOTHESES

Based on the aims presented above it was hypothesized that a spatially distributed low-dose NGF (1µg) injection protocol would cause an immediate acute pain and spontaneous muscle pain, sensitize a larger area of the TA muscle as assessed by pressure algometry and evoke higher contraction pain as compared with a single-site bolus NGF (5µg) injection. Additionally, compared with a non-sensitized TA muscle, it was hypothesized that ischemic muscle contractions performed with an NGF-sensitized muscle after the distributed NGF injections would facilitate ischemic-contraction evoked pain and NGF-induced muscle hyperalgesia. Finally, compared with a non-sensitized TA muscle, it was hypothesized that three repeated low-dose (1µg) NGF injections would induce and maintain muscle pain and muscle hyperalgesia, facilitate ischemic contraction-evoked pain during prolonged NGF-sensitization, and effect central mechanisms such as temporal summation of pain and pain modulation. See also aims and hypotheses of the three studies in *Overview of the studies in Appendix B*

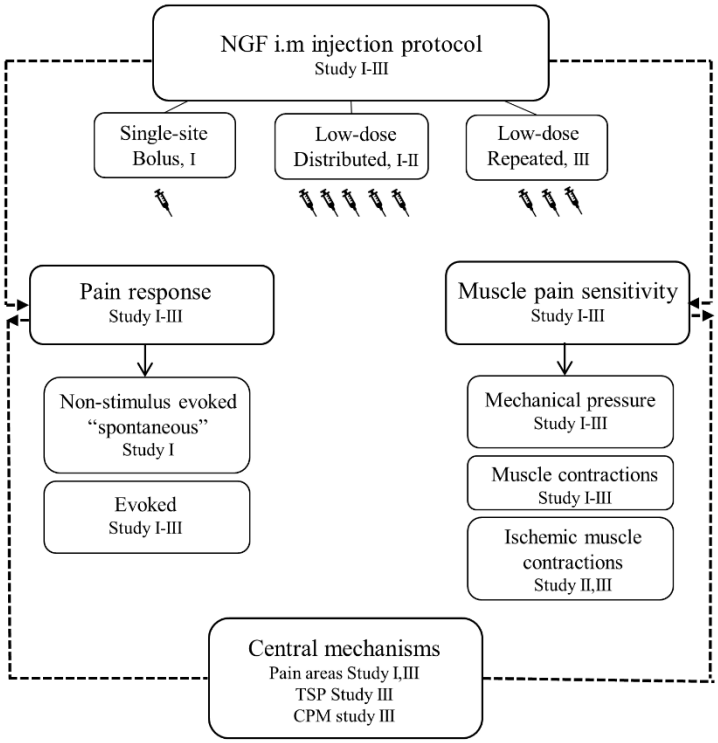

Fig. 1.1 Schematic overview of the dissertation and the included studies. *The studies are defined as Study I, II, and III. The purpose was to explore pain responses (Study I-III), muscle pain sensitivity (Study I-III), and effects on central pain mechanisms (Study III) to novel NGF injection protocols; single-site bolus NGF injection (Study I), low-dose distributed injections of NGF (Study I, II), and repeated low-dose NGF injections (Study III).*

1.5. PAPERS ASSOCIATED WITH THE DISSERTATION

Study I: Sørensen LB, Boudreau SA, Gazerani P, Graven-Nielsen T. Enlarged areas of pain and pressure hypersensitivity by spatially distributed intramuscular injections of low-dose Nerve Growth Factor. Journal of Pain. 2019;20(5):566–76.

Study II: Sørensen LB, Gazerani P, Graven-Nielsen T. Nerve Growth Factor-induced muscle hyperalgesia facilitates ischaemic contraction-evoked pain. European Journal of Pain. 2019;23:1814–1825.

Study III: Sørensen LB, Gazerani P, Sluka KA, Graven-Nielsen T. Repeated injections of low-dose Nerve Growth Factor (NGF) maintain muscle pain and facilitate ischemic-contraction evoked pain. Under peer review in Pain Medicine.

CHAPTER 2. ASSESSING MUSLE PAIN

2.1. PAIN INDUCTION AND ASSESSMENT

Pain is a multidimensional experience and it is therefore important to apply various assessment tools to evaluate different aspects of pain characteristics and the subjective experience of pain (45). In experimental settings, this includes both subjective and semi-objective methods to assess the evoked pain. The current work utilizes psychophysical methods such as rating scale methods and threshold determinations (46) following chemically induced pain provoked by administration of NGF into the tibialis anterior (TA) muscle. The following chapters will present the applied methods and subsequently the main findings.

2.1.1. EXOGENOUSLY EVOKED PAIN

Various methods can be used to induce experimental pain. As such, evoked muscle pain can be induced by electrical, mechanical, and thermal stimuli (9,47,48), and the focus here is on the chemically evoked ones. Indeed, endogenous substances have been used and injected into muscles to evoke a short-lasting experimental muscle pain and hyperalgesia in healthy humans (11). The disadvantage of these substances are their short working time which limits the evoked pain to reflect the somatosensory manifestations of acute clinical muscle pain only (49). Hence, these may not adequately mimic pain that transits from the acute into the long-lasting or persistent pain. Long-lasting pain models have been develop by use of i.m injection of hypertonic saline and capsaicin from manually applying bolus injections to the use of repeated injections and infusion techniques (50); thus, standardizing the delivery and induction of pain across various studies. However, pain is only present for the duration of infusion. In addition, i.m injection of hypertonic saline evokes a strong and immediate pain, although the muscle sensitizing effect is weak and strongly dependent on muscle size (51). On the contrary, single i.m injection of NGF (5µg) evokes a long lasting muscle pain but does not cause any immediate pain during injection or the spontaneous subjective experience of pain post-days injection (35–37). Repeated NGF injections (5µg) prolong the evoked pain and hyperalgesic state (52), with slightly more pain reported following the second and third injections when the muscle is already sensitized. Therefore, as the NGF model evokes long-lasting sensitization, it has been suggested to mimic the time course and processes involved in the transition from acute to more sustained pain better than other chemical models of muscle pain and hyperalgesia (53).

In Study I, five distributed low-dose NGF injections (1µg, 4 cm distance) were administered into the TA muscle and compared with a single-site bolus injection protocol consisting of one NGF injection (5µg), and four injections with isotonic-

saline (of the same volume) administered in the contralateral TA muscle (Fig. 2.1A). The NGF (5µ) bolus was always injected first into the TA belly-site and then followed by the four isotonic-saline injections given at the proximal and distal TA sites. To assess the temporal profile of NGF-induced injection pain in each protocol applied in Study I, pain intensity was continuously evaluated during injections using and electronic visual analogue scale (eVAS) displaying a 10 cm line with the anchors 0 *"no pain"* and 10 *"worst pain imaginable"*. All injections were completed at the end of Day0 after baseline assessments (Fig. 2.2). Spontaneous pain (non-stimulus evoked e.g. pain at rest) was reported in a paper diary during the days post-injection and rated by use of a numerical ranging scale ($NRS_{0\text{-}10}$). Evoked pain with daily activities was assessed by a Likert scale ranging from 0 *"A complete absence of pain"* to 6 *"A severe pain, stiffness or weakness that limits my ability to move"*, and muscle pain sensitivity was assessed over a period of 21-days. In Study II, five distributed low-dose injections of NGF (1µg, 4 cm distance) were administered into the TA muscle in a crossover design with injections of isotonic-saline (control condition) given in the same muscle in the opposite arm of the study (Fig. 2.1A). The injections were completed at the end of Day0 before baseline assessments in each phase, and evoked pain (Likert scale), and muscle pain sensitivity were assessed over seven days (Fig. 2.2). In the last study (III), three single-site repeated injections of low-dose NGF (1µg) were administered into the TA muscle in a crossover design with injections of isotonic-saline (control condition) given in the same muscle in the opposite arm of the study (Fig. 2.1A). The three injections were completed at the end of Day0, Day2, and Day4, and evoked pain (Likert scale), and muscle pain sensitivity were assessed over a period of 21-days (Fig. 2.2).

2.1.2. ENDOGENOUSLY EVOKED PAIN

Human experimental muscle pain can also be evoked with endogenous methods such as ischemia and exercise (i.e. muscle contractions) (54). These methods often include the entire muscle and supporting structures and, therefore, a more widespread and deep muscle pain is evoked as compared with the exogenous techniques (9). Normally, contractions of the muscle are not painful; however, previous studies have shown that pain is evoked during moderate muscle contractions, when the muscle has been sensitized by a prior i.m injection of NGF (35,37). This is a specific feature of the NGF pain model, as contraction-evoked pain has not been demonstrated with other injection-based muscle pain models (55). In study I, it was hypothesized that with more muscle nociceptors being available to be sensitized with the distributed NGF injection protocol (*see Overview of the studies in Appendix B*), larger contraction-evoked pain areas and increased pain would be evoked as compared with the single-site bolus NGF injection. A simple contraction task of the TA muscle was performed after both injection protocols (Fig. 2.1B). Pain intensity was rated verbally on an $NRS_{0\text{-}10}$ after the task, and additionally, contraction-evoked pain areas were drawn on a digital body chart of the lower leg (NavigatePain, Denmark).

Ischemic pain has been widely examined with the tourniquet model (33,56–58), which is an efficient model to evoke intense muscle pain; although the pain may not solely be evoked from muscle tissue but also from the vascular system (59), albeit this is less clear. In Study II and III, a sequence of repeated muscle contractions were performed while a pressure cuff was mounted over the thigh and inflated to occlude blood flow from the working TA muscle (Fig. 2.1B). This was based on a prior human study that examined the effect on muscle pain sensitivity during experimentally ischemic-induced pain in healthy humans (58). In both studies (II, III), pain intensity was rated verbally on an $NRS_{0\text{-}10}$ after the contractions, and compared with the pain intensity evoked by normal muscle contractions (i.e. without the addition of a pressure cuff, Fig. 2.1B) before and after administration of NGF into the TA muscle.

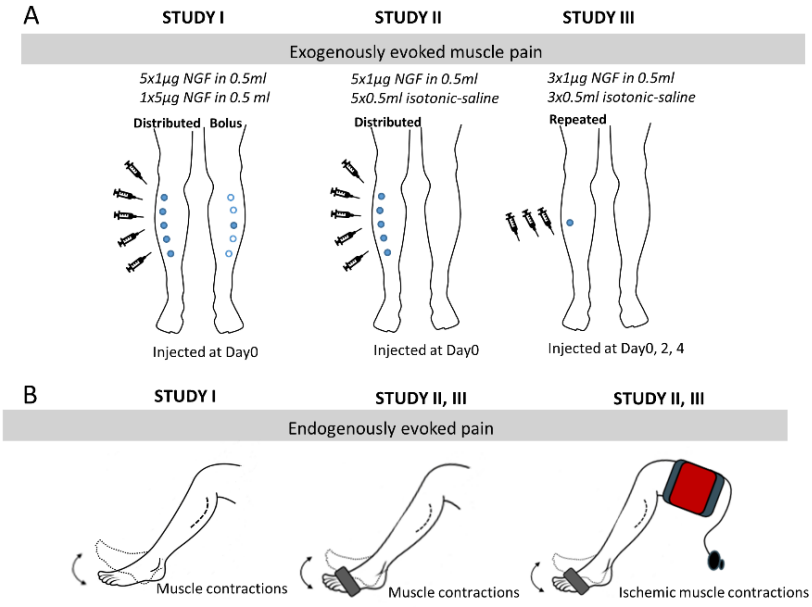

Fig. 2.1 NGF injection protocols and muscle contractions. *A) Illustrations of the 3 NGF injection protocols applied in Study I (single-site bolus NGF (5µg) and distributed low-dose NGF (1µg) injections) in Study II (distributed low-dose NGF (1µg) injections and control injections of isotonic-saline), and in Study III (low-dose repeated NGF (1µg) injections and control injections of isotonic-saline. B) Illustrations of the contraction task performed in Study I and the normal muscle contractions and ischemic muscle contractions (by addition of a pressure cuff) performed in Study II and III*

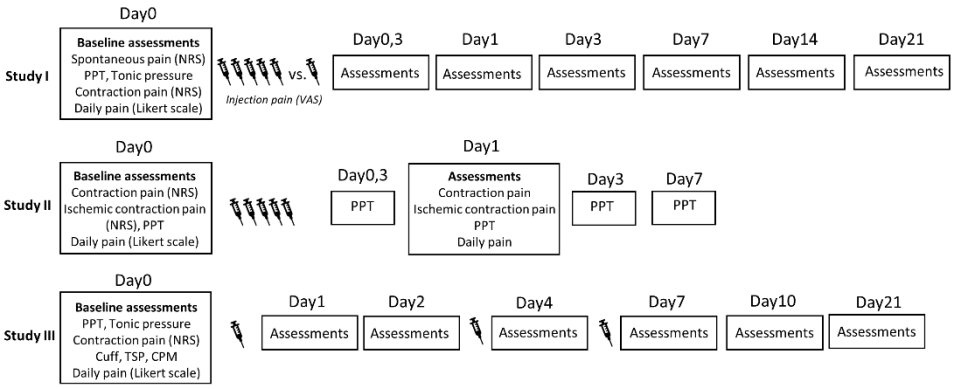

Fig. 2.2 Schematic illustration of the study design*. Assessments and assessments days for each of the 3 studies (I-III). NRS: Numerical rating scale, PPT: Pressure pain threshold, VAS: Visual analogue scale, TSP: Temporal summation of pain, CPM: Conditioned pain modulation*

2.2. COMPARISONS OF PAIN RESPONSES FOLLOWING NGF INJECTIONS

An acute pain response during i.m. injection of NGF was examined in Study I. The VAS pain profile showed a similar time course for both the spatially distributed low-dose NGF (1µg) injections and the bolus NGF (5µg) injection with no clear difference in the pain scores between the two injection protocols (Fig. 2.3). This indicates that the administration of NGF (distributed low-dose vs. single-site bolus) into the TA muscle did not play a significant role on these pain ratings. A prior study showed a low pain intensity upon completion of a single NGF injection (5µg) in the TA muscle (i.e. VAS <0.5/10 cm) (37). In Study I, a similar low pain intensity was shown after the 1st NGF injection (1µg vs. 5µg NGF) in both protocols (VAS ~0.2-0.5/10 cm) with a similar small increase in the pain intensities following the remaining injections (peak-VAS ~2/10 cm) in both NGF protocols. This probably resulted from the greater number of injections given in this study, as only isotonic-saline injections were given after the single-site bolus NGF.

Considering the immediate and acute pain reported after other algogenic substances e.g. hypertonic saline or capsaicin, i.m. of NGF is not considered painful. However, as only, a time-dependent and long-lasting muscle hyperalgesia is developed after NGF injection, this in turn, has allowed studies to examine an acute exacerbation of muscle pain (e.g. injections of hypertonic saline) in a muscle pre-sensitized by NGF (35,52).

No spontaneous pain, i.e. pain at rest, was reported in the days post-injection for both the single-site bolus NGF (5µg) and low-dose distributed NGF (1µg) injections. Although, it was suggested that more muscle nociceptors would be affected by the distributed NGF injections, this was not the case in this study. The lack of spontaneous pain after i.m NGF injection is consistent with prior studies (34,37). However, studies injecting NGF in body tissues with a denser innervation of nociceptors such as skin shows conflicting findings, as acute pain was reported in one study (60) but not in another study (61). Additionally, Rukwied et al. (62) suggested that NGF sensitizes skin nociceptors and increases their responsiveness to an additional inflammatory stimulus (UV-B irradiation), and hence, causing sufficient excitation to induce spontaneous pain in humans. Lastly, no spontaneous pain was reported after NGF injection into the muscle fascia (63), but injection of NGF into the patella fat pad caused a moderate knee pain in few healthy volunteers lasting up to 1-3 month (30).

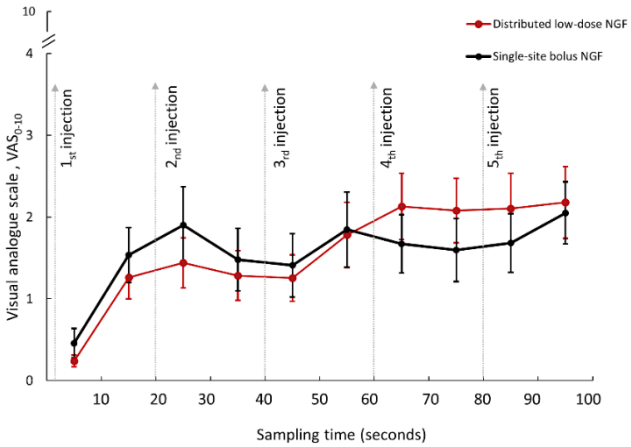

Fig. 2.3 Injection pain. *Mean (±SEM, n=20) VAS scores for the distributed NGF injection protocol (red line) and the single-site bolus NGF injection protocol (black line) assessed during injection in study I. The five injections in each protocol were completed within 90 seconds as indicated by the arrows and pain intensity was continuously rated during this period. VAS: Visual Analogue Scale. SEM: standard error of the mean. NGF: Nerve Growth Factor.*

2.2.1. PAIN EVOKED WITH DAILY ACTIVITY

In accordance with prior human NGF studies (21,34,37), pain was evoked with daily activity (Likert scale) in all three studies (I-III), and caused pain lasting for days (Fig. 2.4). However, the duration and intensity of evoked muscle pain seemed to be dependent on injection protocol. As such, in Study I, muscle pain was reported after Day1 and lasted until Day12 for both the single-site bolus NGF (5µg) injection and low-dose distributed NGF (1µg) injections with the highest Likert pain score shown

approximately 2-days post-injections (Likert scale ~3/6) before the pain intensity started to decline. In the research work by Andersen et al. (37), a single injection of NGF (5µg) into the TA muscle evoked a less intense pain (Likert scale ~2) that was only present until Day7. As both injection protocols (bolus and distributed) were induced at the same time (i.e. one in each TA muscle) in this study, it could be speculated whether the prolonged pain response and higher pain intensities were a combination of both protocols. In Study II, the distributed low-dose NGF (1µg) injections evoked muscle pain after 3 hours that similarly peaked 2-days post injections (Likert scale ~2.5/6) and lasted until Day7 on the last day of testing. In Study III, a less pronounced muscle pain intensity was present 3-days after the low-dose repeated NGF (1µg) injections that peaked approximately 5-days after the first NGF injection (Likert scale ~1.5/6, i.e. 1-day after the 3rd injection), and lasted until Day9. A prior study (21) also showed maintained muscle pain with repeated NGF (5µg) injections, although the peak pain intensity and duration were both higher and longer, respectively (Likert scale ~3/6, lasting until Day16) compared with the repeated low-dose NGF in this study.

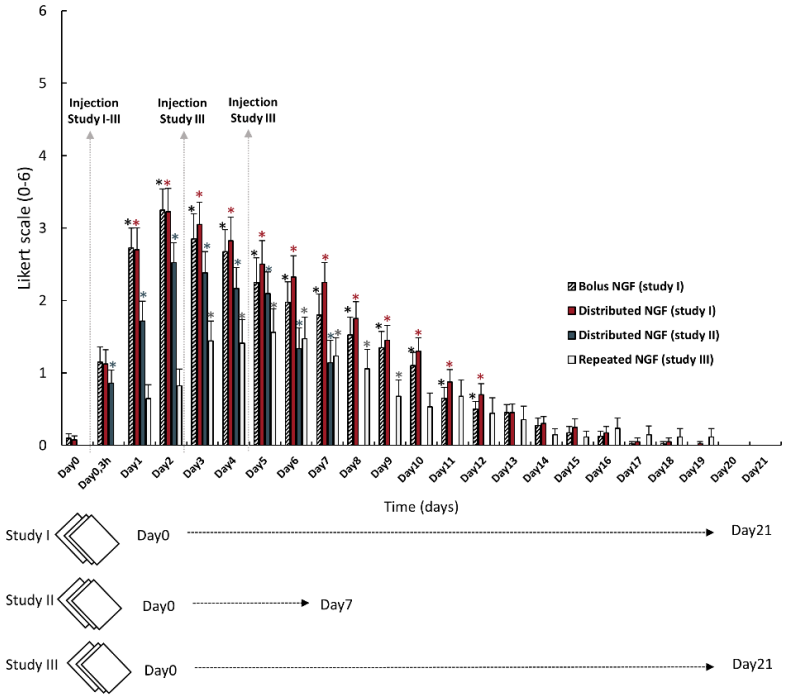

Fig. 2.4 Activity-evoked pain. *Mean (±SEM) Likert scale scores from the paper diary on pain evoked with daily activity in Study I (black: bolus NGF injection, red: distributed NGF injections, n=20), Study II (blue: distributed NGF injection, n=21), and Study III (white: repeated NGF injections, n=17). The evoked pain was evaluated throughout the 21-days of testing in Study I and III, and throughout the 7-study days in Study II.SEM: standard error of the mean. NGF: Nerve Growth Factor.*

2.2.2. PAIN EVOKED WITH NORMAL MUSCLE CONTRACTIONS

Contraction-evoked pain was confirmed across studies (I, III). In Study I, increased contraction-evoked pain was found after both injection protocols (single-site bolus and distributed) lasting until 7-days post injection, with a significantly higher pain intensity at Day3 (peak-pain distributed NGF: NRS ~4.2/10 cm and peak-pain bolus NGF: NRS ~3.4, Fig. 2.5) and larger contraction-evoked pain areas (Fig. 2.6) after the distributed NGF injections. Additionally, in accordance with Andersen et al. (37), the period with increased contraction-evoked pain outlasted the period with increased sensitivity to pressure in Study I, as the PPTs had returned back to normal baseline values at Day7 after both injection protocols. In Study III, contraction-evoked pain was only investigated in the volunteers who responded to the lower dose of NGF (1µg) with muscle hyperalgesia (*see Overview of the studies in Appendix A*). Contraction-evoked pain was significantly increased at Day4 and Day7 (peak-pain NRS ~1.5/10 cm), 3-days after the 3rd NGF injection, when compared with control injection of isotonic-saline (control data not shown in Fig. 2.5).

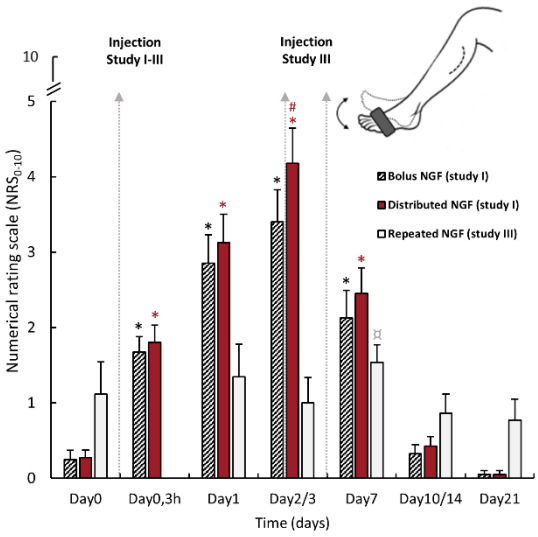

Fig. 2.5 Contraction-evoked pain. *Mean (±SEM) numerical rating scale (NRS) scores after normal muscle contractions performed in Study I) black: bolus NGF injection, red: distributed NGF injections, n=20), and Study III (white: repeated NGF injections, n=13), * denotes different from baseline, # denotes different from bolus NGF injection, ¤ denotes different from control injections (not shown). Time of injections are shown by the grey arrows. SEM: standard error of the mean. NGF: Nerve Growth Factor.*

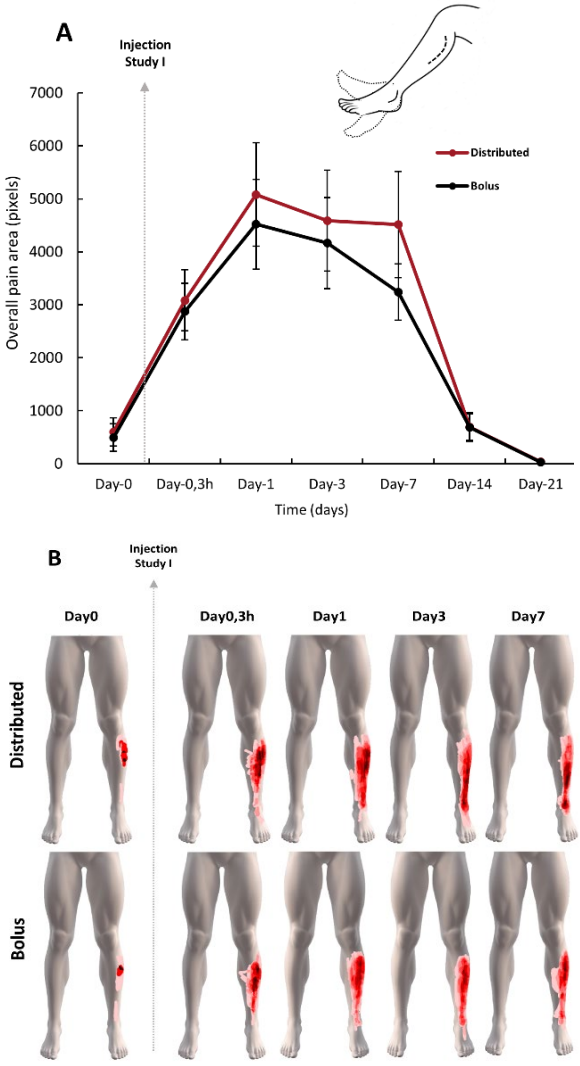

Fig. 2.6 Contraction-evoked pain areas *A) Mean (±SEM, n=20) overall pain areas (black: bolus NGF injections, red: distributed NGF injections), and B) superimposed pain drawings after the contraction task in Study I. Time of injections are shown by the gray arrow. SEM: standard error of the mean. NGF: Nerve Growth Factor.*

2.2.3. PAIN EVOKED WITH ISCHEMIC MUSCLE CONTRACTIONS

In addition to assessing pain induced by normal muscle contractions (Study I), pain evoked with ischemic muscle contraction after NGF injections were assessed in Study II and III. In general, higher evoked pain was reported after the ischemic muscle contractions when compared with the normal muscle contractions after NGF injections in both Studies (II, III). Study II showed that the evoked pain after ischemic contractions was further increased 1-day post NGF distributed injections (NRS: ~7/10 cm. Fig. 2.7). The acute provoked acidification of the TA muscle (i.e. maintained by pressure cuff) did not facilitate NGF-induced pain sensitivity (section 3.2.1 Fig 3.2). Thus it could be speculated whether muscle nociceptors were sensitized by other mechanisms including the activating of local chemo-sensitive channels during such condition, and hence facilitating the increased ischemic contraction-evoked pain at Day1. In study III, it was hypothesized whether prolonged NGF-sensitization maintained by repeated low-dose NGF injections (and a possible effect on central neuronal excitability due to retrograde NGF transport) would further facilitate the ischemic evoked pain over time. As similarly shown in Study II, an increased evoked pain was shown at Day1 after the 1st NGF injection in Study III, although this was not significantly different from the evoked pain at baseline (Day0). Interestingly, the evoked pain after ischemic muscle contractions was significantly increased at Day7 (NRS: ~7.5/10 cm. Fig. 2.7), 3-days after the 3rd NGF injection.

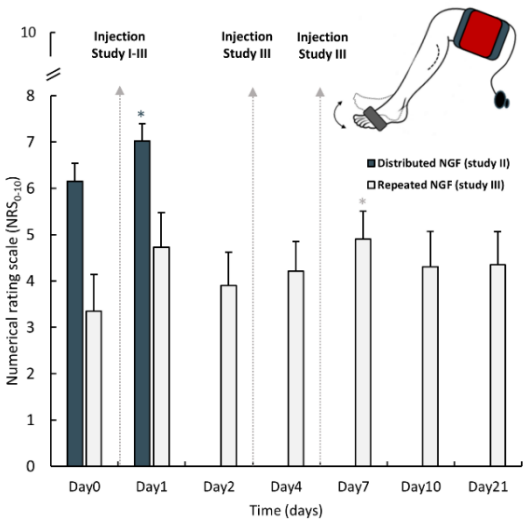

Fig. 2.7 Ischemic contraction-evoked pain*. Mean (±SEM) numerical rating scale (NRS) scores after ischemic muscle contractions performed in Study II (blue: distributed NGF injections, n=21), and in Study III (white: repeated NGF injections, n=13). * denotes different from baseline (Day0). Time of injections are shown by the grey arrows. SEM: standard error of the mean. NGF: Nerve Growth Factor.*

2.3. SUMMARY OF THE MAIN FINDINGS ON PAIN RESPONSES

NGF-induced injection pain was explored in Study I with the single-site bolus NGF injection and low-dose distributed NGF injections. Consistent with prior NGF studies, a single NGF injection (i.e. 1st NGF injection of either 1µg NGF or 5µg in this study) evoked pain <1/10 cm. Additionally, it was speculated whether the distributed NGF injections caused more pain than the single-site bolus NGF as the same total amount of NGF would be distributed over a larger area of the muscle. However, this was not demonstrated in Study I as there was no difference between the two NGF injection protocols during the time of injection. The small increase in pain ratings following the remaining injections probably reflects that the volunteers rated the needle pricks rather than NGF substance, as only isotonic-saline was given after the NGF injection in the bolus NGF injection protocol.

Pain evoked with daily activities was present in all NGF injection protocols. The highest and most enduring evoked pain was demonstrated with both the low-dose distributed NGF (1µg) injections and bolus NGF (5µg) injection in Study I. However, as there was no difference between these two NGF injection protocols, it was speculated whether the evoked pain reflected both at the same time. In Study II, evoked pain was present throughout the period of testing after low-dose distributed NGF (1µ) injections, which also has been shown in a prior NGF study with bolus NGF (5µg) injection. The evoked pain after low-dose repeated NGF (1µg) injections developed later and the pain was less severe compared with both single-site bolus (Study I), and the distributed NGF injections (Study I, II).

Study I showed that contraction-evoked pain was higher in an NGF-sensitized muscle, and additionally, significantly higher contraction-pain and larger evoked pain areas were shown after the low-dose distributed NGF (1µg) injections compared with bolus single-site NGF (5µg) injection. Study II and III showed that contraction-evoked pain was higher when muscle contractions were performed during ischemia (by adding a pressure cuff). In Study II, ischemic-contraction evoked pain was facilitated 1-day post-distributed NGF (1µg) injections. In Study III, ischemic-contraction evoked pain was significantly increased at Day7 after repeated low-dose NGF (1µg) injections and prolonged NGF-sensitization.

CHAPTER 3. MECHANICAL MUSLE SENSITIVITY

3.1. ASSESING MUSCLE PAIN SENSITIVITY

Different modalities can be used to assess pain sensitivity; however, mechanical stimulation, such as mechanical pressure application or manual palpation, is commonly used when assessing deep tissues including muscle pain sensitivity and has been applied in various experimental and clinical studies (64). The mechanical stimulus is applied until a predefined pain response is reached, such as the threshold for detecting the painful stimulus (i.e. pain detecting threshold) or when the stimulus feels intolerable (i.e. pain tolerance threshold). Sensitized muscle nociceptors are good indicators of peripheral sensitization and are characterized by a decreased threshold locally in the affected muscle in response to mechanical stimulation (9). Moreover, enlargements of the affected hyperalgesic area can also be determined by an altered pain sensitivity towards mechanical pressure in more distant areas remote from the original painful site (i.e. referred pain areas) (37).

3.1.1. HANDHELD PRESSURE ALGOMETRY

The most widely used technique to assess muscle pain sensitivity is pressure algometry (65). Although this technique affects both skin and muscle sensitivity (66), deep-tissue nociceptors mediate a major component of pressure induced pain (67), suggesting that the skin may contribute little to the overall pressure pain sensitivity. In all studies (I-III), a manual handheld pressure algometer (Somedic; Hörby, Sweden), equipped with a standard circular probe of 1-cm^2, was used to assess temporal changes in muscle pain sensitivity before and after the injections of NGF at three defined injection sites (most proximal, middle, and most distal) on the TA muscle. Additionally, prior work has shown that NGF affects muscle pain sensitivity at areas outside the site of injection, and at adjacent muscles (37,53). Hence, the extensor digitorum longus (EDL) muscle was assessed in all three studies (I-III). The vastus lateralis (VL) muscle (Study I), and the extensor carpi radialis brevis (ECRB) muscle (Study II, III) were considered as control sites. Pressure was applied at a rate of 30 kPa/s (51), ensuring a steady increase in pressure until the volunteers pressed a stop button, at the point in which they first felt pain (i.e. pressure pain threshold/ PPT). Furthermore, based on the reduction in PPTs at the middle TA site after the distributed low-dose NGF injection protocols in current project (Study I and II), muscle hyperalgesia was defined as a reduction in PPTs of $\geq$ 27% at injection-site from baseline to Day1 in Study III (i.e. responders to the single-site low-dose NGF).

Additionally, in Study II, it was hypothesized that the performance of ischemic muscle contractions in a pre-NGF sensitized muscle would facilitate the NGF-induced muscle hyperalgesia (*see Overview of the studies in Appendix B*). Hence, muscle pain sensitivity was assessed immediately after ischemic contractions while the cuff pressure was maintained (1st bout) and was measured again at post cuff deflations (immediately post and 10 min post).

3.1.2. CUFF PRESSURE SENSITIVITY

In addition to the manual pressure algometer, pain detection threshold (PDT) and pressure pain tolerance (PTT) determinations were assessed bilaterally over the lower legs in Study III, by a computer-controlled pressure cuff system consisting of two 13 cm wide pressure cuffs (VBM Medizintechnik GmbH, Sulz am Neckar, Germany) and an $eVAS_{0\text{-}10}$ (Aalborg University, Denmark). In contrast to manual pressure algometry, cuff pressure stimulates a larger tissue volume (68), and hence affecting a higher proportion of afferent fibers from the deep tissues (69). During cuff algometry, the pain intensity related to the inflation of the cuff is used to establish a stimulus-response curve that allows for assessing deep-tissue pain sensitivity in both healthy humans (68,70) and patients (71,72). In Study III, the cuff pressure was increased by one kPa/s, with a maximum pressure at 100 kPa. Pain intensity was continuously rated on the eVAS when the pressure first became painful (i.e. PDT), and until the pressure pain could no longer be tolerated by the volunteer (i.e. PTT), where then a stop button was pressed.

3.2. COMPARISONS OF PRESSURE PAIN SESNITIVITY FOLLOWING NGF INJECTIONS

In all three studies (I-III), localized NGF-induced muscle hyperalgesia was developed in the TA muscle with each NGF injection protocol (Fig. 3.1A, B, C). Development of muscle hyperalgesia and duration of increased muscle sensitivity after both single-site bolus NGF (5µg) injection and distributed low-dose (1µg) NGF injections were comparable with a prior NGF study (37), where decreased PPTs were assessed after 3 hours and lasting until Day3 in Study I and II. Additionally, in Study I, decreased muscle sensitivity was assessed at 21-days after injections in both NGF protocols. Such increases in PPTs has also been shown in other long-term studies with repeated pressure stimulations after i.m NGF injections (34,37) but also in a non-sensitized muscle (73). Furthermore, decreased muscle sensitivity was not shown after prolonged period of NGF-sensitization with the repeated NGF injections in this study. In Study III, 4 out of 17 volunteers were defined as not responding to the low-dose NGF injection with muscle hyperalgesia (*see Appendix B*). In the remaining 17 volunteers, decreased PPTs were shown 1-day post the 1st injection that were maintained until Day7 after repeated low-dose NGF injections (i.e. 3-days after the 3rd injection). Although, these 17 volunteers were defined as responding to the low-dose NGF, the reduction in PPTs after repeated NGF injections was less pronounced

than the reduction in PPTs after the NGF injection protocols applied in Study I and II (Fig. 3.1A, B, C). Furthermore, there was no further reduction in the PPTs after the 2nd and 3rd NGF injection in Study III, as previously demonstrated in the studies with repeated NGF (5µg) injections (21,74). Work by Hayashi et al. also showed prolonged NGF-induced muscle hyperalgesia after three days consecutive repeated NGF (5µg) injections that developed after the 1st injection and lasted until Day6 (i.e. 3-days after the 3rd NGF injection).

Additionally, in Study II and III, PPTs were also decreased after control injections of isotonic-saline (control data not shown in Fig. 3.1). It could be speculated whether this reflected a certain expectation of NGF effect (i.e. placebo effect (75)) as both Study II and III were performed as cross-over studies. However, a prior NGF study showed an increased muscle sensitivity in the control muscle i.e. isotonic-saline injection, although the decrease from baseline values was smaller than after NGF injection (34). In Study II, the overall decrease in PPTs across sessions were lower after the distributed low-dose NGF injections. However, this could not be shown with repeated low-dose NGF injections in Study III.

Enlargement of the hyperalgesic area outside the injection-sites could not be determined with the low-dose distributed NGF injections in Study I and Study II. However, decreased PPTs at the proximal and distal assessments sites (8 cm distance) were recorded after single-site bolus NGF injection in Study I that were present up to 3-days after the NGF injection. Additionally, the proximal and distal sites also showed decreased PPTs after repeated low-dose NGF injections in Study III, lasting until Day7 (Fig. 3.1A, C). A time-dependent spreading of the hyperalgesic area has been shown up to 4 cm and 8 cm remote from injection-site in prior studies after both single NGF (5µg) injection (37) and repeated NGF (5µg) injections (21). A more widespread effect of NGF was investigated with the EDL muscle, as this muscle shares the same neural innervation with the TA muscle (i.e. deep peroneal nerve). Decreased PPTs were recorded after 3 hours until Day1 at the EDL muscle in Study I after both NGF injection protocols (bolus and distributed), and after the distributed NGF injections in Study II. There was no effect on muscle sensitivity at the EDL muscle in Study III after repeated NGF injections (Fig. 3.1D). Lastly, no NGF-induced effect on muscle pain sensitivity was recorded at the control VA muscle in Study I. However, the ECRB muscle showed decreased PPTs 1-day post distributed NGF injections in Study II, which possibly could stem from repeated pressure stimulation in the smaller muscle. In contrast, no changes in PPTs over time were recorded at ECRB in Study III after repeated NGF injections (Fig. 3.1E). As NGF most likely does not cause any effects on extra-segmental body sites in humans, the VA and ECRB muscles seemed suitable as control assessment sites for the TA muscle in the current study.

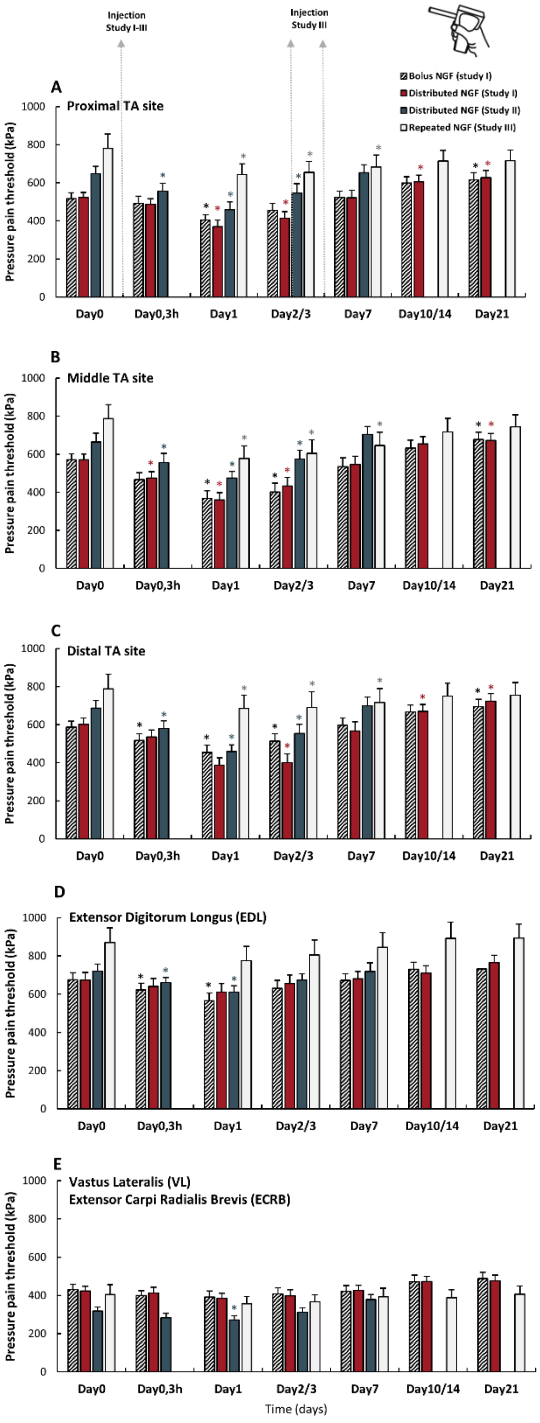
Injection Study I-III
Injection Study III
Bolus NGF (study I)
Distributed NGF (Study I)
Distributed NGF (Study II)
Repeated NGF (Study III)
A
Proximal TA site
B
Middle TA site
C
Distal TA site
D
Extensor Digitorum Longus (EDL)
E
Vastus Lateralis (VL)
Extensor Carpi Radialis Brevis (ECRB)
Pressure pain threshold (kPa)
0
200
400
600
800
1000
Day0
Day0,3h
Day1
Day2/3
Day7
Day10/14
Day21
Time (days)

***Fig. 3.1 Pressure pain thresholds (PPTs)**. Mean (±SEM) PPTs assessed at the; (A) proximal TA site, B) middle TA site, C) distal TA site, D) the Extensor Digitorum Longus (EDL), E) Vastus Lateralis (VL), and Extensor Carpi Radialis Brevis in Study I (black: bolus NGF injection, red: distributed NGF injections, n=20), Study II (blue: distributed NGF injections, n=21), and Study III (white: repeated NGF injections, responders: n=13). *, denotes difference from baseline. SEM: standard error of the mean. NGF: Nerve Growth Factor.*

3.2.1. EFFECT OF ISCHEMIA AND ISCHEMIC MUSCLE CONTRACTIONS ON MUSCLE PAIN SENSITIVITY

In Study II, increased PPTs at all TA injection sites were shown immediately after cuff deflation at Day0 (pre-injection), which was further increased at Day1 (post-injection) immediately after cuff deflation and up to 10 min post cuff deflation (Fig. 3.2) when NGF had sensitized the TA muscle. Hypoalgesia to pressure has been observed after 15 min with maintained ischemia (i.e. differential nerve block) (76), immediately after isometric exercise (77), and up to 5 min after an isometric contraction task of the exercised leg (67). In Study II, ischemic muscle contractions were performed in two bouts with a break of 3 min in between. PPTs were recorded after the 1st bout only, and the 2nd bout was used to maintain the evoked pain while the ischemic condition was maintained for 6 min in total. Therefore, the effect of ischemic muscle contractions on muscle pain sensitivity could possibly be explained by a combination of normal inhibitory processes resulting from the moderate pain evoked by ischemic exercise (e.g. exercise-induced inhibition (EIH) (78,79)).

Although it was hypothesized that an acute acidification of the TA muscle would facilitate muscle pain sensitivity, this could not be demonstrated in Study II. Therefore, the ischemic muscle contractions may sensitize muscle nociceptors through other mechanisms. This may possibly include the activation of chemo-sensitive channels, as the ischemic contraction-evoked pain was increased. However, as mentioned above, also mechanisms of afferent inhibition might have been activated that shortly could mask the NGF-induced muscle hyperalgesia upon cuff deflation.

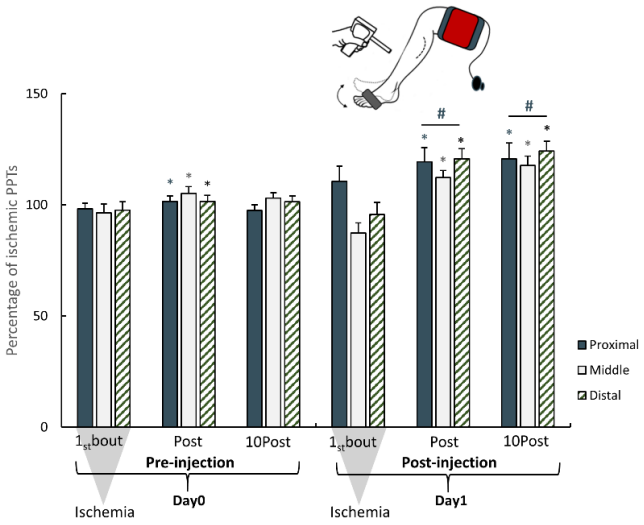

Fig. 3.2 Ischemic effects on pressure pain thresholds (PPTs). *Mean (±SEM, n=21) PPTs recorded over the TA muscle (green: proximal TA site, white: middle TA site, and striped: distal TA site) in Study II after ischemic muscle contractions and maintained pressure cuff (1st bout), and at post cuff deflations (immediately post and 10 min post). PPTs were normalized to the first recording before ischemia at Day0 (pre-NGF injections), and Day1 (post NGF injections). * denotes difference from first recording at either Day0 or Day1. # denotes difference from Day0. SEM: standard error of the mean. NGF: Nerve Growth Factor.*

3.2.2. EFFECT OF NGF ON CUFF PRESSURE PAIN SENSITIVITY

In Study III, there was no effect on PDTs and PTTs over time with repeated low-dose NGF injections. Often, thresholds values adapt to repeated pressure stimuli and hence, increase with each subsequent assessment, which has been shown in healthy humans without the induction of experimental pain (68) and during experimentally-induced muscle pain (80) using cuff algometry. Moreover, the latter study (80) also showed lack of changes in threshold values between assessments days, probably indicative of competing processes of tissue sensitization and adaptation (81). This might also explain the lack of changes in Study III, as threshold values were assessed over the sensitized muscle in healthy volunteers with an otherwise normally functioning pain system.

3.3. SUMMARY OF THE MAIN FINDINGS ON MUSCLE PAIN SENSITIVITY

A local and time-dependent muscle hyperalgesia developed in the TA muscle after all NGF injection protocols (Study I-III). In Study I and II, muscle hyperalgesia was present after 3 hours and lasted until Day3 after single-site bolus NGF (5µg) and low-dose distributed NGF (1µg) injections. In Study III, few volunteers (4 out of 17) did not respond to the low-dose NGF injections with muscle hyperalgesia. However, in volunteers, defined as responding to the low-dose NGF injection, a less pronounced muscle hyperalgesia developed 1-day after the low-dose repeated NGF (1µg) injection protocol that lasted until Day7 (3-days after the 3rd injection). Proximal and distal TA assessments sites were affected by single-site bolus NGF (5µg) injection in Study I and after repeated low-dose NGF (1µg) in Study III. Additionally, a more widespread effect of NGF was assessed at the EDL muscle after single-site bolus NGF and low-dose distributed NGF injections (Study I, II), but not with the low-dose repeated NGF injections in Study III. In general, VA and ECRB muscle seemed not to be affected by injection of NGF (Study I and III), and therefore suitable for control assessment sites in this study.

In Study II and III, the NGF injection protocols (low-dose distributed and low-dose repeated NGF injections) were controlled by injections of isotonic-saline in the opposite study arm. Both studies showed increased muscle pain sensitivity (i.e. decreased PPTs) after control injections, which possibly could stem from certain expectations to NGF effect, as both studies were performed as a crossover design. However, in Study II, the PPTs were generally lower (i.e. more decreased) after distributed low-dose NGF injections than the PPTs recorded after the control injections.

Study II demonstrated that acute endogenous evoked ischemia in the TA muscle did not facilitate NGF-induced muscle hyperalgesia. Instead, decreased muscle pain sensitivity was shown after ischemic muscle contractions post cuff deflation at Day0 (pre-NGF injection) that was further increased immediately after cuff deflation and 10 min post cuff deflation at Day1, when NGF already had sensitized the muscle. Other mechanisms such as the effect of exercise and the ischemic block (i.e. pressure cuff) could possibly mask the effect of NGF-induced hyperalgesia shortly after cuff deflation.

Study III showed no effect on cuff pressure sensitivity between the days of testing after repeated low-dose (1µg) NGF injections. The lack of changes over time could probably result from competing processes of NGF-induced sensitization and the adaptation to repeated stimuli that normally occurs.

CHAPTER 4. ASSESSING CHANGES IN RELATION TO CENTRAL PAIN MECHANISMS

4.1. CENTRAL SENSITIZATION

Over the past decade, prior human NGF studies have shown that NGF induces changes in the pain signaling systems that cannot exclusively be explained by the induction of peripheral sensitization and appears to have a central component. As such, single-site i.m injection of NGF (5µg) induces larger areas of muscle hyperalgesia 1-day post injection (21,37), affects muscle pain sensitivity in neighboring muscle (53), maintains muscle hyperalgesia and pain, and facilitates the enlargement of pressure-induced pain areas and temporal summation of pain after consecutive repeated NGF (5µg) injections (21). Evidence shows that NGF is internalized in axons of peripheral nerve endings that express the high affinity receptor, tropomyosin receptor kinase A (trkA) and retrogradely transported to the cell body in the DRGs (19,20,82). Here, NGF modulates the expression and levels of various proteins e.g. Calcitonin gene-related peptide (CGRP), Brain-derived neurotropic factor (BDNF), substance P, TRPV1, and ASICs (17) with a possible effect on neural excitability in these sensory afferents (83) as reflected by an increased release of these substances at central terminals with neural activity (84). Hence, NGF actions on sensory afferents gene expressions play an important step in the generation of altered central pain processing (85). With the slow retrograde transport (86) and the time required for NGF-induced gene regulation, the changes related with a central component would presumably show a different time course compared with the local effect induced by NGF (87). This demonstrates the importance of conducting longer duration experimental investigations in NGF studies. In study I and III, the effect of NGF was assessed over a period of 21-days since muscle hyperalgesia tends to return to normal baseline values at 7-days post single i.m NGF injection (36,37) and around Day10, after repeated NGF injections (21). The following section will introduce the methods for assessing changes in relation to central pain mechanisms used in this Ph.D. study (I, III) and present the main findings.

4.2. MAINTAINED PRESSURE STIMULATION

Maintained pressure stimulation has been used experimentally to study pain referral patterns by nociceptive stimulation of the upper neck muscles such as the infraspinatus muscle, which were comparable to the pain referral patterns observed after i.m injection of hypertonic saline (88). The reasons underlying pain referral and widespread pain are presumably linked to a central mechanism, as referred pain can

be evoked in areas where sensory inputs are blocked (89), although a peripheral component cannot be ruled out entirely (4). Research work by Doménech-García et al. (88) demonstrated that pain areas provoked by tonic pressure stimulation of the infraspinatus muscle expanded with prolonged muscle soreness (i.e. DOMS), and additionally, Hayashi et al. (21) showed that expanded pain areas to tonic pressure stimulation on the TA muscle developed progressively with repeated NGF injections (i.e. prolonged NGF-sensitization). Therefore, to evaluate the effect of supra-threshold pressure stimulation linked to a possible change in central mechanisms e.g. pain referrals or widespread pain, a 30-s tonic pressure stimulation was applied with the handheld pressure algometer on the TA muscle in Study I and III. In Study I, three injection sites (proximal, middle, and distal) were assessed and pressure was applied at 120% of the PPTs recorded in each respective experimental session. Areas of pain following the stimulation subsequently were drawn on a digital body map of the lower leg (NavigatePain; Aalborg, Denmark). In Study III, pressure was applied to the middle TA site (injection-site), and pressure was applied using the same methodology as in Study I.

4.3. TEMPORAL SUMMATION OF PAIN

Effects of a central faciliatory component on muscle pain intensity can be assessed by repeated nociceptive inputs. As such, muscle pain intensity has shown to be facilitated in some musculoskeletal pain conditions (90,91) and following experimental pain models in humans (92,93). This phenomenon is known as Temporal summation of pain (TSP), and is suggested to mimic the phenomenon of wind-up that occurs in dorsal horn neurons in animals (94), which plays a fundamental role in the generation of pain hypersensitivity (95). Repetitive mechanical pressure stimulation on muscles with a fixed stimulus intensity has been used in various experimental set-ups to assess TSP (96–98). Recent studies have demonstrated that TSP is facilitated during DOMS (93), in a combined NGF (5µg) and DOMS pain model (36), and with maintained NGF-sensitization after repeated NGF (5µg) injections. In Study III, TSP was assessed with the automated cuff algometry and eVAS systems over the injected TA muscle. A series of 10 sequential pressure stimulations (1s duration, 1s interval) were applied with the same stimulus intensity as the PTT recorded in the same session, and the pain intensity was continuously rated on the eVAS during the period of stimulation.

4.4. CONDITIONED PAIN STIMULATION

Finally, the effect of a central modulatory component was also tested in Study III after repeated low-dose NGF (1µg) injections, which has not been investigated during prolonged NGF application. Such pain modulatory mechanisms were originally based on the observation that responses of dorsal horn neurons to noxious stimuli were inhibited by acute noxious stimulus from an extra segmental site (i.e. diffuse noxious inhibitory control/DNIC) (99). Conditioned pain modulation (CPM) is the method

used to explore DNIC-like effects in humans, and typically tests the difference in the response to a test stimulus (i.e. pressure detection threshold) before and during or after the presence of a painful conditioning stimulus (e.g. tonic mechanical or thermal stimulation) (100). In healthy humans, the effect of CPM is typically shown by reduced pressure pain sensitivity in response to the painful conditioning stimulus (101,102). In a prior NGF study (37), reduced pressure pain sensitivity was assessed after 14-21 days post-NGF (5µg) injection after the muscle sensitivity was back to normal baseline values. Whether this resulted from e.g. adaptation to the test-procedures (i.e. repeated pressure stimulation) or a slower normalization of the descending pain controls systems are unknown. Therefore, in Study III, effect of CPM was assessed by the automated cuff algometry, and the conditioning stimulus was induced by inflation of the pressure cuff placed on the contralateral leg (103) (i.e. non-injected TA muscle) and maintained a constant pressure at 70% of PTT during the test. A second cuff placed over the injected TA muscle, slowly induced pressure with a rate of 1 kPa/s, and PDT and PTT were reassessed. CPM-effect was quantified as the difference between PDTs with and without the conditioning stimulus.

4.5. CENTRAL EFFECTS OF NGF-INDUCED PAIN AND MUSCLE SENSITIVITY

There was no enlargement of the pressure-induced pain areas at either injection-site (proximal, middle, and distal) in Study I after both the single-site bolus (5µg) NGF injection and the distributed low-dose (1µg) NGF injections, nor was there any difference between the two injection protocols (Fig. 4.1A, B, C). Additionally, in Study III, there was no change in the size of local pain areas with repeated low-dose (1µg) NGF injections assessed at the middle TA injection-site (Fig. 4.1D). Moreover, in Study III, the area of pressure pain was not different from control condition (isotonic-saline injections Fig. 4.1D). Compared with the methodology used by Hayiashi et al. (21) and the higher pressure-intensity induced at each experimental session, the intensity of tonic pressure stimulation was based on the PPTs recorded at each respective session, and hence, induced at a lower pressure intensity in Study I and III. Additionally, studies have shown a correlation between the nociceptive stimulus (i.e. pain intensity) and the area of referred pain (88,104), thus the low-pressure intensity applied in Study I and III could probably be an explanation for the lack of changes in the size the local pressure-induced pain areas.

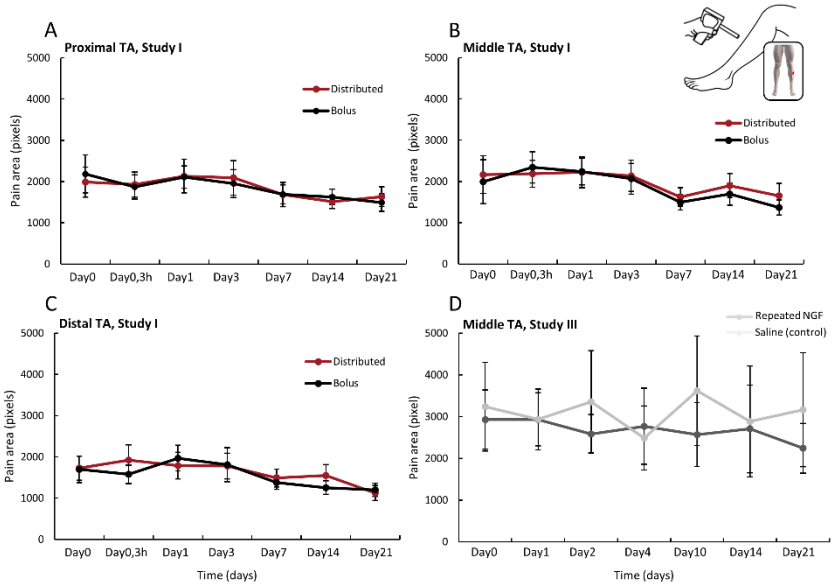

Fig. 4.1 Pressure-induced pain areas*. Mean (±SEM) size of local pain area following the tonic pressure stimulation for the A) proximal TA site, B) middle TA site, C) distal TA site in Study I (black: bolus NGF injection, red: distributed NGF injections, n=20), and D) the middle TA site in Study III (grey: repeated NGF injections, light grey: isotonic-saline, n=13). There was no difference in local pain area size after either NGF injection protocol or after isotonic-saline injections. SEM: standard error of the mean, TA: tibialis anterior, NGF: Nerve Growth factor*

4.5.1. EFFECT OF MAINTAINED NGF-INDUCED MUSCLE HYPERALGESIA ON TSP AND CPM

In Study III, central changes related with both anti-nociceptive and pro-nociceptive mechanisms (i.e. CPM and TSP) were investigated during repeated NGF injections. There were no change over time in CPM-effect, suggesting that the descending inhibitory pathways were not affected by prolonged NGF-induced sensitization in this study (Fig. 4.2A). In experimental studies, an impaired CPM has been shown during painful saline-induced muscle pain (102), and during prolonged noxious pain evoked by topical capsaicin in healthy volunteers (105), suggesting that CPM alterations may be dependent on the pain intensity (106,107). In Study III, a less intense muscle pain was evoked with repeated NGF injections (Likert scale: ~1.5/6), which might have been insufficient to significantly affect CPM.

Cuff pressure-induced TSP did not change with prolonged NGF-sensitization (Fig. 4.2B), which contrasts the findings in prior NGF studies, in which NGF-induced facilitation of TSP was demonstrated 1-day post NGF injection (21,98). However, compared with the low induced muscle pain, evoked with the low-dose repeated NGF injections (Likert scale: ~1.5/6), pain facilitation resulted from a combined NGF and DOMS evoked muscle pain (Likert scale: ~3.5/6) presented in research work by Nie et al. (98).

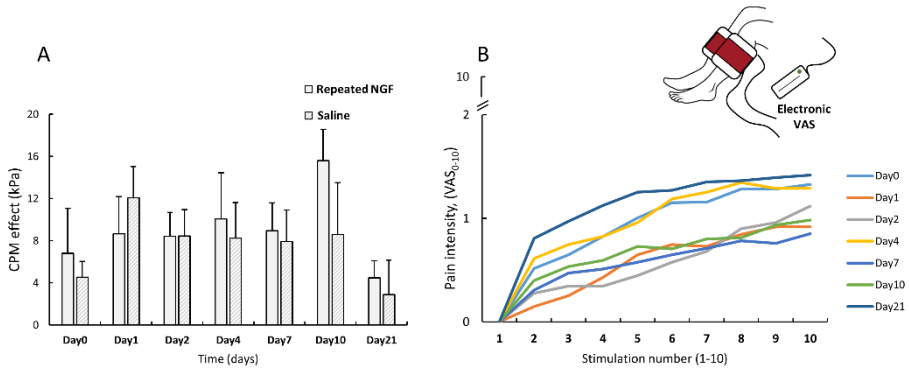

Fig. 4.2 Conditioned pain modulation and temporal summation of pain*. Mean (±SEM) A) Conditioned pain modulation (CPM)-effect (PDT with minus without conditioning) assessed in Study III (white: repeated NGF injections, striped isotonic-saline, n=13) and B) Temporal summation of pressure pain (TSP) assessed in Study III at Day0 (pre-injections), and during repeated NGF injections at Day 1, 2, 4, 7, 10, and 21. There was no effect on repeated NGF injections on CPM-effect and TSP. SEM: standard error of the mean, NGF: Nerve Growth Factor, VAS: visual analogue scale*

4.6. SUMMARY OF THE MAIN FINDING OF CHANGES IN RELATION TO CENTRAL PAIN MECHANISM

Study I showed no effect of maintained tonic pressure stimulation on the size of local pain areas after single-site bolus NGF (5µg) injection or with distributed low-dose NGF (1µg) injections in Study I, and similarly, no enlargement of pressure-induced pain areas was shown after repeated low-dose NGF (1µg) injections with maintained NGF-sensitization in Study III. This could possibly be explained by the relatively lower pressure pain intensity given at post NGF injection-days, as intensity was based on the PPTs measured in each respective session.

Study III showed no temporal changes on CPM-effect and cuff-pressure induced TSP during maintained NGF muscle hyperalgesia, suggesting that the low-dose repeated

NGF (1μg) injections did not significantly alter the modulatory and faciliatory components of the central inhibitory pain systems in current study. The pain intensity may be an important factor as CPM is impaired during noxious stimulation such as hypertonic-saline injection and capsaicin applied on healthy volunteers, and TSP is facilitated during combined DOMS and NGF-induced pain. Therefore, the muscle pain intensity evoked by the low-dose repeated NGF injection protocol may not be considered enough severe to significantly affect CPM and TSP in this study.

CHAPTER 5. CONCLUSION AND FUTURE PERSPECTIVES

The current Ph.D. thesis has addressed specific objectives and clarified significant details about the NGF pain model by aid of novel NGF injection protocols. Pain responses during and after i.m NGF application, as well as evoked pain with daily activities and muscle contractions were explored in all NGF injection protocols. Effects of NGF distribution on muscle pain sensitivity were studied using methods that assessed changes related with both peripheral and central mechanisms in these NGF protocols. The main findings obtained from all three studies were presented in previous chapters, and an overview is provided in Fig. 5.1.

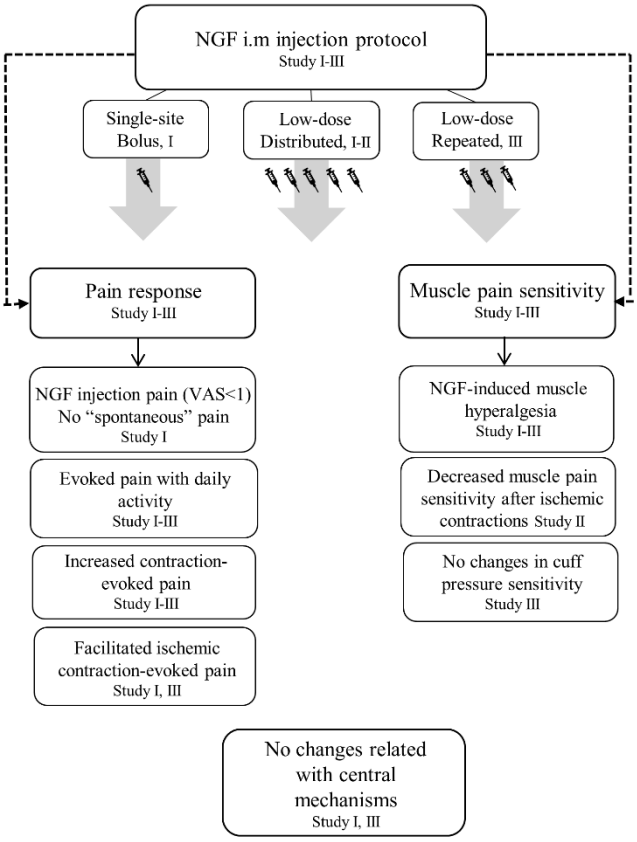

Fig. 5.1 Dissertation outline with the main findings *presented from the three studies (I-III)*

It was hypothesized that NGF pain response was dependent on a spatially distributed delivery into the TA muscle and a lower dose to avoid potential overdosing locally at injection site. However, Study I showed that no acute injection pain or spontaneous pain (pain at rest) post injection-days was developed after distributed low-dose NGF (1µg) injections or a single-site bolus NGF (5µg) injection. The distributed NGF injections assumedly sensitized a larger area of muscle tissue compared with the single-site bolus NGF injections, and hence higher evoked pain and a larger pain area after muscle contractions were demonstrated with the distributed NGF injections. Additionally, the low-dose distributed NGF injections explored in Study I seemed equally adequate for inducing pronounced muscle hyperalgesia and activity-evoked pain as for the single-site bolus NGF injection.

NGF seems important molecule in generating and facilitating evoked pain responses. This could likely stem from NGF-sensitized muscle nociceptors that increase the responsiveness of other active components of the pain processing system such as the chemo-sensitive TRPV1 channels. Study II showed that pain evoked after ischemic muscle contractions (by adding a pressure cuff) was facilitated 1-day post distributed low-dose NGF injections. In contrast, NGF-induced muscle hyperalgesia was not facilitated by acute acidification of the TA muscle milieu, but probably was affected by other mechanisms e.g. related to exercise-induced inhibition during the pain evoked by ischemic muscle contractions as decreased pain sensitivity to pressure was seen after the contractions as post cuff-deflation.

Finally, NGF was presented to cause changes in pain distribution and facilitated pain responses in humans that are related with a central component that cannot be explained solely by local peripheral mechanisms, although this might still be controversial. NGF maintained nociceptor activity is suggested to stem from the process of retrograde transport of NGF when NGF is present in the tissue for a longer time, and a subsequent altered central processing due to de novo gene expression and receptor modulation at central nerve endings. Study III showed that low-dose repeated NGF injections maintained a less pronounced muscle hyperalgesia in volunteers that responded to the low-dose NGF. However, the NGF-induced muscle hyperalgesia lasted only 3-days after the 3rd NGF injection, which was similar to the duration of muscle hyperalgesia induced with the single-site bolus NGF protocol and distributed NGF injections in Study I and II. Additionally, ischemic contraction-evoked pain was increased at Day7 after prolonged NGF-sensitization. However, the low-dose NGF injections evoked pain to a much lesser extent (peak Likert scale: ~1.5/6) than the pain evoked with both NGF injection protocols in Study I and II and hence, might not sufficiently alter CPM and facilitate TSP.

In conclusion, the current PhD study has extended the prior work on NGF human pain models and has provided significant additional details to the current knowledge.

This is an important step forward and the insights gained from the present work offer advantages for future experimental studies of prolonged muscle pain and muscle hyperalgesia using naturally occurring substances such as NGF that are likely to be present during the pathological muscle pain and involved in muscle nociception. Additionally, a great amount of research work has explored the sensory manifestations of muscle pain sensitivity and evoked pain in the TA muscle following e.g. chemically-induced pain, exercise-induced pain (13,104,108–111), and NGF i.m injections (21,30,37), and the TA muscle seems practical feasible for studying the novel NGF injection protocols utilized in this current study.

From a mechanistic aspect, comparisons with other NGF-injected muscles may be necessary to gain more knowledge on NGF-induced muscle sensitivity. Both the trigeminal innervated muscle such as the masseter muscle (34,112), and upper limp muscle e.g. wrist extensors muscle (35,52,74), and neck and shoulder muscles (36,53) have now been investigated in several human studies (*Appendix A*). Recently, it was shown that the relative increase in muscle pain sensitivity at injection-site did not depend on muscle type when the PPTs were compared between four different muscles (trapezius, supraspinatus, ECRB, TA) 1-day post-NGF (5µg) injection (113). Based on the prior NGF studies (*Appendix A*) and the current findings, the duration of NGF-induced muscle hyperalgesia seems to last approximately 3-days after single-site bolus NGF (5µg) injection and low-dose (1µg) distributed NGF injections, that can be prolonged with repeated NGF injections, indicative for an applicable use of the NGF pain model in various muscle types. Although, the entire muscle compartment is affected by the distributed low-dose NGF injections, a disadvantage of the distributed NGF injection protocol are the greater number of injections required compared with the single-site bolus NGF injection. Although no response-dose relationship has been performed in human NGF pain models, the single-site and repeated low-dose NGF injection protocol may open up some questions as if future studies should investigate the issue of responder and non-responders subgroups. However, as shown in the present study the single-site low-dose repeated NGF injection protocol may not sufficiently mimic aspects of muscle pain and pronounced muscle hyperalgesia.

Additionally, future studies may look into perspectives on NGF evoked pain to clarify further the involvement of central-mediated mechanisms in NGF pain models. Few studies have explored how the long-lasting effect of NGF-induced muscle sensitization modulates motor neuroplasticity with evoked muscle pain in the masseter and ECRB muscles using the single bolus NGF (5µg) injection and repeated NGF (5µg) injections (74,114) which has not been explored in leg muscle.

Even though induction of experimental pain and pain assessments were performed in humans in this study, a mechanistic approach based on e.g. animal findings is still encouraged in future human studies to gain further insights into peripheral and central-mediated mechanisms related to muscle pain and NGF-induced sensitization,

although a link between mechanisms in humans must always be cautiously interpreted. The current findings illustrate the role of NGF in muscle pain and hyperalgesia and highlight its influence on peripheral afferents affecting a larger area of the muscle and an altered response during ischemic conditions. Therefore, the novel low-dose distributed NGF model explored in the current work may mimic some aspects of muscle pain and peripheral muscle sensitization with clinical implications in e.g. local ischemic pain conditions. Additionally, alterations in muscle milieu seem important activators of peripheral sensitization (115,116) that drive the pain during inflammatory conditions. Hence, recordings from the muscle milieu by aid e.g. of microdialysis technique during NGF-sensitization and muscle acidification could be explored in future studies. Furthermore, applications of pharmacological tools to manipulate some of these peripheral pathways under question in this dissertation could be of interest. Recently, it was shown that local i.m injection of delta-9-tetrahydrocannabinol (THC), and non-psychoactive cannabinoids attenuated NGF-induced sensitization in rat masseter muscle (117,118), thus potentially leading the steps to explore the potential of cannabinoid-based medicine in future NGF pain models.

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APPENDICES

Appendix A. Overview of human NGF studies

Appendix A shows and overview of human NGF studies and summarizes their main findings on NGF-evoked pain responses and muscle pain sensitivity in relation to both peripheral and central mechanisms.

Literature searches have been performed throughout the thesis period using databases such as PubMed primarily, and secondarily Google Scholar and the reference list from other studies. A range of different keywords and terms has been used alone or in combination during the literature search. These are the following;

Nerve Growth Factor, NGF, muscle hyperalgesia, NGF sensitization, muscle pain, ischemic contraction, ischemic muscle pain, repeated injections,

Study	Clinical study; safety and efficacy	Design and aims	Pain induction	Assessments	Main findings
Petty et al. 1994	Model of neuropathy	- 45 healthy subjects (12F) - Phase 1 study - Double-masked, placebo-controlled, single-dose and randomized study - Monitored at hospital for 24h after inj. and returned after 2, 4, 8 weeks AIM: Evaluate safety and tolerance assessment in healthy subjects Determine if single doses would cause formation of antibody or changes in nerve conduction parameters	i.v NGF (0.03-1 µg/kg) s.c NGF (0.03-1 µg/kg) Placebo	General health status assessed through standardized assessments incl. - Nerve conduction - QST Blood sampling (plasma NGF, serum antibodies) Adverse experiences Lab abnormalities	NGF doses > 0.1µg/kg: mild-moderate diffuse myalgia after 60-90 min – resolving over 2-8dy. Duration and severity varied in a dose-dependent manner. At NGF doses (0.03-0.01) no serious adverse symptoms developed i.v → abdominal spread of pain areas s.c → inj. site hyperalgesia to heat and touch. Skin described "extra sensitive" No spontaneous pain, No antibody found Plasma levels (NGF) only detectable in subjects receiving 1.0 µg/kg i.v after 5 min sampling. Neurological exam was normal at 2-week follow-up
Study	**Experimental pain (deep tissue)**	**Design and aims**	**Pain induction**	**Assessments**	**Main findings**
Svensson et al. 2003	Temporomandibular disorders + Myofascial pain	- 12 healthy male subjects - Double-blind, randomized and placebo-controlled study - Repeated follow-up: BL + 1h, 1, 7, 14, 21, 28dy post inj. AIM: Testing effect and duration of mechanical sensitivity changes after NGF injection in muscle	i.m NGF (5µg/ 0.2 ml) i.m saline (0.2ml) (masseter muscles)	Mech. sensitivity: - Pressure pain threshold (PPT) - Pressure tolerance threshold (PTOL) Oral function: RDC/TMD questionnaire: rate pain on NRS0-10 at various oral functions + at rest	No spontaneous pain Local signs of mech. allodynia and hyperalgesia at dy1 to 7dy + pain during strenuous jaw movement Sig. mechanical allodynia at dy 1 following saline although the decrease was smaller than compared with the decrease in PPTs after NGF
Svensson et al. 2008	Temporomandibular disorders (TMDs)	- 14 healthy male subjects - Double-blind, randomized and placebo-controlled study -Repeated follow-up: BL + 1, 2, 3, 24h post inj. AIM: Testing if NGF-induced sensitization is associated with changes in vibration sense and stretch reflex sensitivity, and facilitation of glutamate-evoked pain	i.m NGF (5µg/ 0.2 ml) i.m saline (0.2ml) (masseter muscle) i.m glutamate (1M/0.2ml) in masseter after 24h	Pain intensity (VAS0-10) Pressure pain threshold (billat.) Vibration sense (NRS): - Effective stimulus for large-diameter mechanoreceptive afferents and muscle spindle Jaw stretch reflex McGill + pain drawings Sensitivity to glutamate inj.	NGF did not cause more pain than saline. NGF: PPTs ↓ 1, 2, 3, 24h post inj. Vibration and amplitude of jaw reflex were not affected by NGF-sensitization. Glutamate ↓ PPTs after NGF and saline. No further decrease in PPTs in NGF pre-treated muscle. No diff. in perceived pain intensity from glutamate inj. in pre-treated NGF or saline muscle – pain areas was larger in NGF-treated muscle NGF inj. is associated with a distinct and prolonged sensitization to mech. stimuli, without effect on large-diameter mechanoreceptive and muscle spindle afferents

Nie et al. 2008	NGF model (facilitating effects) Single NGF model vs. Combined model (NGF+DOMS)	- 10 healthy male subjects - Randomized and placebo-controlled - Repeated follow-up: BL + 3h, 24h, 4, 7, 21dy post inj. AIM: Explore if NGF intensifies DOMS pain and whether TSP of pressure pain is facilitated during hyperalgesia induced by NGF or NGF combined with DOMS.	i.m NGF (5µg/ 0.2 ml) i.m saline (0.2ml) (trapezius muscle) DOMS (3h post inj)	Pressure pain threshold (PPT) DOMS soreness (VAS) Max. voluntary contraction (MVC) Pressure stim. (TSP)	NGF + DOMS pain ↑ 3h + 24h post inj. PPT ↓ 3h + 24 post inj. NGF: evokes higher pain than DOMS alone NGF intensified DOMs responses + facilitates evoked temporal summation of pain
Andersen et al. 2008	NFG-induced hyperalgesic distribution	- 20 healthy subjects (10F) - Randomized and placebo-controlled - Repeated follow-up: BL + 3h, 1, 4, 7, 21dy post inj. AIM: Explore spatial distribution of NGF muscle hyperalgesia (local + spread) and pain quality of chemical excitation in hyperalgesic area	i.m NGF (5µg/ 0.2 ml) i.m saline (0.2ml) (tibialis ant. muscle) i.m HS (5.8%/0.5ml) in both TA after 24h	Likert scale diary (activity pain) Mech. testing: Pressure pain thresholds (PPTs) Tactile, skin (von Fray) Muscle hardness (contraction, VAS) Chemical pain (HS, VAS) McGill (quality of HS provoked pain)	NGF: PPTs ↓ after 3h until D3 post inj. Proximal + distal expanded hyperalgesic areas (1-4dy post) HS changed the pain quality NGF induced muscle soreness during activity (3h to7dy post inj.)
Gerber et al. 2011	Therapeutic testing on NGF-induced hyperalgesia	- 11 healthy subjects (2F) - Randomized and placebo-controlled - Repeated follow-up: BL + $D0_{IA}$, D1, $D1_{IA}$, D7 post inj. AIM: Explore effect of current treatment: (local block) Detect/exclude muscle tissue as primary source in conditions involving central mech.	i.m NGF (5µg/ 0.2 ml) (supraspinatus, bilat.) i.m ropivacaine (0.25%, 6-10ml) /saline after 24h	Pain intensity (rest and movement, VAS) Mech. sensitivity: - cutaneous (von Fray) - Pressure pain sensitivity (PPTs) Shoulder exercise (time until pain) Injection pain (VAS)	NGF-induced delayed hyperalgesia (24h post inj) to adjacent muscles (infraspinatus) Pain and spreading are maintained despite anesthesia. Local anesthesia is not a valid diagnostic procedure
Deising et al. 2012	NGF-induced fascial pain model	- 14 healthy male subjects - Randomized and placebo-controlled - Repeated follow-up: BL + 1h, 1, 3, 7, 14, 21dy post inj. AIM: Explore time course of local hyperalgesia in fascia and NGF-sensitizing effect on ASICs channels + TRPV1 by phosphate buffer (pH4)	Fascial inj: NGF (1µg/ 100µl) Saline (100µl) (Erector spinae at L4-L5) Day 4+7: inj. of phosphate buffer (ph4) (TRPV1 sensitization)	Mechanical impact pain (VAS) Mechanical tonic pressure stimulation Pressure pain thresholds (PPTs) Electrical-induced muscle contractions Heat pain thresholds Pain to buffer injection	No acute pain NGF provokes long-lasting sensitization of nociceptors within the fascia (erector muscle) → mech. stim. 6h to D7 NGF ↑ proton-induced pain at D7+D14 within buffer administration
Hayashi et al. 2013	Repeated injection model	- 12 healthy subjects (5F) - Randomized and placebo-controlled - Repeated follow-up:	i.m NGF (5µg/ 0.2 ml) x3 i.m saline (0.2ml) x3 (tibialis ant. muscle)	Injection pain (VAS) Muscle pain (Likert scale) Pressure pain thresholds (PPTs)	Higher inj. pain (<1/10cm) after 2nd and 3rd inj. Likert scale: pain ↑ 1-16dy post inj. PPTs: ↓ 1-3dy post inj. No further reduction

	(Reflecting the process in MCP conditions e.g. myofascial trigger points)	BL + 1h, 1, 2, 3, 6, 10dy post inj. AIM: Explore progressive effects of induced-repetitive NGF and central and peripheral mech. potentially effective in trigger points		Cutaneous sensitivity (von Fray) Sequential pressure stim. (TSP) Tonic-induced pressure stim. + drawing of pain area	PPTs ↓ 8cm from injection site at Day2-3 Facilitated TSP: 1-10dy post inj. Pain areas: ↑ 1-6dy post injection Excluding skin as contributor to hyperalgesia
LoVecchio et al. 2014	Combined model: UVB-irradiation in skin above NGF-sensitized muscle	- 25 healthy subjects (11F) 1. Experiment: (arm + low back) Effect of heat rekindling on UVB-irradiated area + mapping area of sec. hyperalgesia (n=9, -7dy, 0dy, 3dy) 2. Experiment: (low back + trapezius); Control site, UVB alone, NGF alone, UVB+NGF. Effect of heat rekindling on UVB alone and UVB+NGF + mapping allodynia (n=16, -7dy, 0, 1, 2, 3dy)	i.m NGF (5µg/ 0.2 ml) i.m saline (0.2ml) (billat. erector spinae muscles (L4 level) UVB-irradiation: Individual MED (UVB)	Muscle pain (Likert scale) Mapping area of hyperalgesia: Pin pricks (pain threshold) Mapping area of allodynia: Von Fray (change in sensation)	Exp. 1: 3dy post UVB: areas of sec. hyperalgesia in arm and low back ↑ after 1. rekindling and maintain after 2. rekindling. Exp. 2: ↑ muscle pain in all subjects (Likert scale) at D1. UVB and UVB+NGF: areas of allodynia develop after 1dy until 3dy. After rekindling: allodynic areas of both UVB and UVB+NGF sites enlarge. UVB irradiation induces both areas of allodynia and hyperalgesia in skin independent on application site. NGF-induced muscle sensitization did not override or potentiate the effect of UVB
Weinkauf et al. 2014	Comparing NGF-induced sensitization patterns in lumbar and tibial muscle and their fascia	- 16 healthy male subjects - Randomized and follow-up: BL + 0.25, 1, 3, 7, 14, 21dy post inj. AIM: explore if temporal sensitization patterns may depend on the distance between NGF inj. site and the spinal ganglia of the targeted nociceptors + Difference in pain response between back muscle and leg muscle	n=8: NGF (1 µg/100µl) tibialis ant. + fascia n=8: (1 µg/100µl) erector spinae muscle (L4 lumbar level) and fascia Citrate buffer inj. (pH 4) at Day7 and 14	Mechanical sensitivity: -Spatial extend (algometry, distance) - Intensity (tonic pressure, VAS) Heat pain threshold Electrical-induced muscle twitch Chemical pain intensity in pre-treated sites (VAS)	Responses: maximal between 1-7dy after NGF inj. at both test sites - and not dependent on distance between NGF injection sites and spinal ganglia. PPTs ↓ continuously within 1 week: Mechanical pressure induces stronger response in NGF-treated fascia than muscle. NGF spreads more easily in fascia - NGF in muscle may be more locally deposited. Inj. of buffer pH 4: ↑ pain in fascia. Pain intensity was not different between d7 and d14
Schabrun et al. 2015	Model adaptations in the transition of sustained muscle pain (repeated inj.)	- 12 healthy subjects (8F) - Repeated follow-up: BL + 2, 4, 14dy post inj. AIM: explore time course of M1 organization and function in response to progressively developing muscle soreness (repeated NGF inj.)	i.m NGF (5µg/0.2ml) x 2 (right ECRB muscle) i.m HS (5.8%/0.5ml) at Day4	PRTEE Muscle pain (Likert scale) Pain quality (Short-form McGill) Grip force (MVC) Pressure pain thresholds (PPTs), billat. Corticomotor excitability; - EMG activity from ECRB (MEPs) - TMS (single-pulse) - Motor cortical maps	Localized muscle pain (Likert scores) and PRTEE ↑ 2-4dy post injection Grip force ↓ D4, and maintained at dy14. PPTs ECRB: ↓ D2-4 (↓PPTs at control arm on D4) Inj. of HS in NGF-sensitized muscle: - Increases MEP amplitude - Reduce ICF - No changes in SICI Altered M1 organization and impaired function characterized by increased corticomotor excitability

				- Intercortical inhibition (SICI)/ facilitation (ICF) - Interhemisperic inhibition (IHI)	(increased map volume, map peaks, reduced SICI, increased ICF) present at D4 and potentiated by acute pain (HS).
Bergin et al. 2015	Model of sustained elbow pain	- 26 healthy subjects (7F) - Randomized and double-blind - Repeated follow-up: BL + 2, 4, 10dy post inj. AIM: Explore the time course and pain location by NGF injection in ECRB and study pain effects on sensory -motor function	n=13: i.m NGF (5µg/ 0.2 ml) n =13: i.m saline (0.2ml) (ECRB muscle) HS inj. at Day2 (chemical irritation)	Muscle pain (Likert scale) PRTEE Pain distribution (drawing) Pain diary (Likert + drawings) Contraction/stretch (strength) Grip force (MVC) + NRS Pressure pain thresholds (PPTs) HS injection pain (VAS) + contraction	No pain at rest Likert: ↑ 12h - D6 post injection NGF group: greater pain (VAS) than control group NGF group: larger pain areas than control NGF group: greater pain evoked by MVC NGF group: PPTs ↓ D2-D4 HS injection: evoke more pain in NGF group
Munkholm et al. 2017	Combined model of NGF and acid stimulation Provocation in a sensitized system	- 32 healthy male subjects - Randomized and double-blind 1 Experiment: n=16 (IFP) 2 Experiment: n=16 (TA muscle) - Repeated follow-up: BL + 1h, 2h, 3h, 1_{pre}, 1_{post}, 2dy post inj. AIM: Explore interaction between NGF-sensitization and acid provoked pain in two NGF models	i.m NGF (5µg/ 0.2 ml) i.m saline (0.2ml) (IFP + TA muscle) Acidic phosphate-buffered saline (5-10ml, pH: 5.4)	Pressure pain thresholds (PPTs) Pain (Likert scale) Acid injection pain (eVAS)	Exp. 1: Persistent pain 1-3 month in the NGF-treated knee. Acid did not facilitate the NGF-induced hyperalgesia Peak pain VAS: 8-9 min Exp. 2 Acid facilitates the NGF-induced hyperalgesia Peak pain VAS: 5-7 min
DeMartino et al. 2018	Model of lateral epicondylalgia (combined model of repeated NGF and DOMS)	- 24 healthy subjects (14F) - NGF group - NGF+DOMS group - Randomized and follow-up: BL + 2, 4, 6dy post inj. AIM: Explore whether NGF+DOMS evoke greater responses than NGF alone	i.m NGF (5µg/ 0.2 ml) x3 (ECRB muscle) DOMS pain on D4	Muscle pain (Likert scale) Pain distribution (drawings) PRTEE EMG activity from ECRB (MEPs) SEPs non-painful nerve stim. Grip force (MVC) Pressure pain thresholds (PPTs)	Muscle pain (Likert) worsens by DOMS - ↑ D2-4 in both groups - ↑ further D 4-6 in NGF+DOMS PPTs: ↓ D2-6 in both groups NGF+DOMS provoked more soreness, larger pain areas and more disability, and decreased force. No diff in somatosensory changes between the groups NGF inj. extended ECBR motor map volume, which was depressed by DOMS
Costa el al. 2019	Myofascial pain: relationship between pain and motor function	- 42 healthy subjects - NGF group (n=25) - Saline group (n=17) - Randomized, double-blind, placebo, follow-up: BL + D2	i.m NGF (5µg/ 0.2 ml) i.m saline (0.2ml) (masseter)	EMG activity from masseter (MEPs) TMS (motor map volume) Jaw pain (NRS) Jaw function (Likert scale, JFLS-20, and oral behaviour checklist)	i.m NGF caused jaw pain on chewing and affected jaw function. NGF group: ↓ map amplitude and volume D2 Saline group: ns modulation effect

		AIM: Explore the modulatory effect of NGF on corticomotor excitability + the correlation to clinical aspects of jaw pain and function. HYP: that NGF would provoke a ↓ in corticomotor excitability			Correlation between cortico excitability and jwa pain + function → higher the ↓ in excitability, the lower jaw pain on chewing (negative corr.)
Study	**Experimental pain (superficial tissue)**	**Design and aims**	**Pain induction**	**Assessments**	**Findings**
Dyck et al. 1997	Skin hyperalgesia	- 16 healthy subjects (3F) - n=8 received 1µg NGF - n=8 received 3µg NGF - Repeated follow-up: BL + 3h, 1, 3, weekly until symptoms or abnormal findings disappear	i.d NGF (1-3µg) i.d saline (same volume) (mid-volar forearm)	The Neuropathy Symptoms and Change (NSC): 38-items Tactile allodynia: - Cotton wool test - puff of air test Pressure allodynia (finger test) Distribution of tenderness (drawing) Vibratory detection threshold: - by CASE IV Cooling detection threshold/ Heat-pain threshold Antibody for NGF before inj. and 18dy after	No experience of systemic symptoms or lab abnormalities. No detectable antibodies to NGF. Side effects of NGF inj.: - Severe headache - upper abdominal tightness/ nausea and generalized myalgias for 2 dy. ↑ frequencies of neuropathic symptoms after NGF vs. saline-injected area at 1, 3, 7, 14, 21dy No tactile allodynia Pressure allodynia at NGF site: 3h, D3, 7, 14 and remained at D21. NGF site: ↓ heat-response: at 1, 3, 7dy post inj.
Paterson et al. 2009	Skin inflammation	- 10 healthy female subjects - Randomized AIM: Study the release of NGF from non-neuronal cells and BDNF from nociceptive neurons in normal and exp. sensitized skin + upon nociceptor stimulation	Skin sensitization (daily shaving the axilla: 7dy) Citric acid (pH 3) / saline perfusion	Inflammation (Laser Doppler Imaging): area of erythema Heat pain threshold Dermal microdialysis + ELISA	Inflammation (↓ heat thresholds at the axilla sites + worsened acid stim.) No diff. in protein content between baseline and acid stimulation. NGF + BDNF: no diff. between body sites (alterations over time) ↑ levels in the acidic phase in all sites – ↑ levels in the inflamed axilla up to 30 min.
Rukwied et al. 2010	Skin hyperalgesia Experimental model of neuropathic pain	- 16 healthy subjects (8F) - Randomized and double-blind - Repeated follow-up: BL + 1, 3, 7, 21, 49dy post inj. AIM: Explore spatial and temporal profile of NGF-evoked hyperalgesia and allodynia to thermal, mechanical,	i.d NGF (1µg/50µl) i.d saline (50µl) (central volar forearm)	Injection pain (NRS) Vasodilation (Doopler) Nociceptor sensitization: - mech. (touch, pinprick) - thermal (heat, cold) - electrical (current pulse) - Axon reflex flare	No signs of inflammation, itch or spontaneous pain NGF heat pain ↓ 1-21dy post inj. Max. mech. hyperalgesia evoked at D21 and lasted over 7 weeks when thermal sensitization has subsided. Cold hyperalgesia: at D7 + 21 Axon reflex flare: unaffected Sensitization limited to inj. site

		electrical stimuli (local vs. central components)			
Rukwied et al. 2013	Combined model of NGF-sensitization with an acute inflammatory model (UVB irradiation)	- 13 healthy male subjects - Randomized and follow-up: BL + 22, 24, 28, 35, 49, 70dy post inj. AIM: Explore if NGF-sensitized nociceptors increased the responsiveness to inflammatory mediators (spontaneous pain)	i.c NGF (1µg/50µl) x2 (anterolat. aspect of both lower legs) 4 tests sites: - native skin (control) - NGF-sensitized skin - UVB irradiated skin - NGF/UVB treated skin 3x MED: 21dy post inj.	Individual MED (before inj.) Spontaneous pain within 1-84h after irradiation (NRS, quality descriptors, related events) Brush-allodynia: D22 Impact stimulation Tonic pressure + pin prick Heat pain threshold Supra-threshold heat Vasodilation (Doppler)	9/13 subjects experienced a mild spontaneous/ evoked pain in NGF/UVB skin Combined NGF/UVB intensified hyperalgesia → additive for the impact stimuli at D22 The occurrence and intensity of spontaneous pain followed the time course of inflammatory UV-B sensitization.
Rukwied et al. 2013	Spontaneous pain model (NGF+UVB)	- 13 healthy male subjects - Randomized and follow-up: BL + 21, 22, 24, 28, 35, 49, 70dy post inj. AIM: Explore axonal hyperexcitability to electrical stimuli and study whether this would correlate with sensory hyperalgesia and spontaneous pain	i.c NGF (1µg/50µl) x2 (anterolat. aspect of both lower legs) 4 tests sites: - native skin (control) - NGF-sensitized skin - UVB irradiated skin - NGF/UVB treated skin 3x MED: 21dy post inj.	Individual MED (before inj.) Electrical stimulation: - perception threshold - electrical pain threshold - supra-threshold stimuli + VAS - Habituation to electrical stimuli Vasodilation (Doppler)	Perception threshold (electrical pulses) did not sig. change. NGF + UVB: ↑ electrical pain above control levels (NGF: up to 49dy), (UVB: up to 72h). Pain levels for NGF/UVB site ↑ for all stimulation frequencies above level recorded for single sites at D22+24, Axon reflex: ↑ in NGF/UVB site at 24h vs. control and single sites No correlation between measures of spontaneous pain (Rukwied 2013) and electrically induced pain (this study). NGF/UVB: correlation between electrically induced pain and supra-threshold pain to pin prick and heat given at 24h after UVB irradiation
Weinkauf et al. 2015	Skin hyperalgesia Differential time course between different modalities and chemical responsiveness	- 8 healthy male subjects - Randomized and double-blind - Repeated follow-up: BL + 3h, 6h, 1, 2, 3, 5, 7, 10, 14, 21dy post inj. AIM: Explore temporal NGF hyperalgesic development at paraspinal sites (short axonal transport) + quantify chemical responsiveness to cinnamon aldehyde (TRPA1 agonist)	i.d NGF (1µg/50µl) x2 i.d saline (50µl) (into the L4/L5 processi spinose skin) Cinnamon aldehyde (20%,60µl) filter paper at D3 and D21 post inj.	Electrical stimulation: - detection threshold - pain threshold - suprathreshold Mechanical impact stimuli (VAS) Static mech. pressure and pinprick: - static pressure (VAS) - Pressure pain threshold - Pinprick (perception) Thermal threshold - warmth detection - heat pain Vasodilation (vascular responses)	The temporal profile of hyperalgesia was similar to previous NGF responses (= short distance to DRG did not matter). Heat sensitization: <3h and peaked between D3-7. Mech. and electrically induced hyperalgesia at D2-3, peaked at D10-14, maintained until D21. Pain upon Cinnamon aldehyde: ↑ at NGF sites vs. control site → linking instant heat hyperalgesia to TRPV1 phosphorylation and translocation.

Obreja et al. 2018	Characterization of nociceptive patterns in single afferents within and outside an NGF injected area	- 11 healthy subjects (8F) - Randomized and double-blind - Recordings of Human C nociceptors 3 weeks after NGF inj. AIM: Explore how NGF alters the excitability of primary afferents fibers in humans + provide evidence for local sensitization within the receptive field of single nociceptors	NGF (0.2µg/10µl) x5 Saline (0.9%/10µl) (into the area of the peroneal nerve innervation territory of the left foot dorsum.	Mech. pin prick hypersensitivity: (area of sensitized skin) Microneugraphy: From cutaneous c-fiber. Receptive fields located by trans-cutaneous electrical search stimuli. Action potentials (AP) recorded at ankle level -2 branches from same parent axon (The collision technique) -Responsiveness of C-fibers to mech., thermal, sympathetic stimulation (The masking technique) - dose-response function of current strength and duration - post excitatory effects - nerve fiber classification	A clear separation of axonal charmechano-sensitive (CM) and mechano-insensitive (CMi) → lost in the NGF foot. At NGF inj. sites: -lower electrical thresholds - reduced ADS (signals of axonal hyperexcitability) - adaption to supra-threshold mech. Less responses + after-discharges were recorded → local sensitization = a combination of sensory sen. and axonal hyperexcitability may underlie localized mech. hyperalgesia at NGF site. Priming in nociceptors: Changes in the axonal properties of nociceptors surrounding the NGF injected zone – not accompanied by hyperalgesia

Appendix B. Overview of the studies included in the thesis

Table presenting the aims, objectives, and conclusions of the three studies included in the thesis.

	Study I	Study II	Study III
Aim	To compare a spatially distributed low-dose (1µg) NGF injection protocol with a single-site bolus NGF (5µg, same total dose) injection in the TA muscle and assess pain responses and muscle hyperalgesia	To provoke acute acidification of the TA muscle by ischemic muscle contractions in a NGF-sensitized muscle, and explore the effect of pain responses and muscle hyperalgesia compared with a non-sensitized TA muscle (control injections of isotonic-saline)	To explore if 3 repeated low-dose NGF induce muscle hyperalgesia in the TA muscle, and if muscle hyperalgesia is maintained; investigate prolonged effect of NGF on pain responses and central pain mechanisms compared with a non-sensitized muscle (control)
Rationale	• Bolus NGF injection locally deposited to the may saturate the tissue (overdose) at that area, and hence not be sufficient for inducing spontaneous pain. Distributing low-dose NGF over a larger area of the muscle tissue could potentially lead to spontaneous pain • The distributed NGF injections affect the whole length of the muscle. Hence a larger area of NGF-induced muscle hyperalgesia is induced. However, it is unknown whether contraction of the TA muscle would cause a larger contraction-evoked pain area and more evoked pain, compared with the single-site Bolus NGF	• Acute acidification of the muscle milieu can be induced with ischemic muscle-contractions that potentially open up chemo-sensitive channels (TRPV1, ASICs) • If increased sensitivity of these chemo-sensitive channels is facilitated by NGF, this might affect muscle pain sensitivity • NGF increases the pain evoked by muscle contractions, however it is unknown whether the evoked pain would be facilitated when NGF has sensitized the muscle tissue, and muscle contractions are performed during ischemia	• Maintained muscle hyperalgesia may be related with a central component through retrograde NGF transport and altered central processing • If NGF-induced sensitization is prolonged with repeated low-dose NGF injections, it is unknown if ischemic contraction-evoked pain facilitates over time, and additionally if changes related with central mechanisms such as pain distribution, CPM and TSP are affected
Objectives and hypotheses	Compared with a single-site bolus NGF injection, spatially distributed low-dose NGF injections cause: 1) immediate and spontaneous pain 2) sensitize a larger area of the TA muscle as assessed by pressure algometry (hyperalgesia) 3) evoke higher intensity and area of contraction-evoked pain	Compared with a non-sensitized TA muscle (control), ischemic muscle contractions performed with a NGF-sensitized muscle after distributed NGF injections induce: 1) facilitation of ischemic contraction-evoked pain at Day1 2) facilitated NGF-induced muscle hyperalgesia	Compared with a non-sensitized TA muscle (control), 3 repeated low-dose NGF injections induce: 1) prolonged pain and muscle hyperalgesia 2) facilitation of ischemic contraction evoked pain 3) enlarge tonic-pressure induced pain areas 4) facilitate TSP and modulate CPM
Conclusion	Study I showed that distributed low-dose NGF injections caused a similar muscle pain and hyperalgesia as shown for the bolus NGF injection. Additionally, larger area of contraction-evoked pain and higher contraction-evoked pain intensity were shown after the distributed NGF injections as compared with the single-site bolus NGF injection.	Study II showed that acute ischemic contraction-evoked pain is higher than pain evoked with normal muscle contractions, and further facilitated at Day1 when NGF has sensitized the TA muscle. Ischemic muscle contractions may sensitize muscle nociceptors e.g. by activating chemo-sensitive channels, thus increasing the ischemic contraction-evoked pain – and additionally, ischemic contractions may shortly mask the NGF-induced muscle hyperalgesia by other mechanisms including afferent inhibition during such contraction task as decreased muscle sensitivity was shown upon cuff deflations.	Study III showed that repeated low-dose NGF injections induced low muscle pain and less pronounced muscle hyperalgesia that was maintained in volunteers responding to the low NGF dose. Additionally, prolonged NGF-induced sensitization increased ischemic contraction-evoked pain at Day7, but did not affect changes related with a central pain component (pain distribution, TSP, and CPM).
Clinical perspective	The distributed NGF injection protocol may better portrait aspects of clinical muscle pain with larger area of sore muscle tissue that becomes more painful with movement of the affected muscle	Interaction between peripheral muscle sensitization and ischemic evoked pain may be important aspect, considering the role of e.g. NGF in peripheral muscle sensitization in clinical ischemic pain conditions	

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