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Minocycline reduces experimental muscle hyperalgesia induced by repeated nerve growth factor injections in humans: A placebo-controlled double-blind drug-crossover study

Running head: Minocycline reduces muscle hyperalgesia

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Significance: In a double-blind placebo-controlled drug-crossover study, the common antibiotic minocycline was found to reduce the muscle hyperalgesia induced by intramuscular injection of nerve growth factor. The results of the study showed that both concomitant (pre-emptive) and delayed administration of minocycline can ameliorate the onset and facilitate the resolution of experimentally induced muscle hyperalgesia.

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Abstract

Background: Hyperalgesia is a heightened pain response to a noxious stimulus and is a hallmark of many common neuropathic and chronic pain conditions. In a double-blind placebo-controlled drug-crossover trial the effects of concomitant and delayed minocycline treatment on the initiation and resolution of muscle hyperalgesia were tested.

Methods: An initial cohort (n=10) received repeated injections (5 µg: days 0, 2 and 4) of nerve growth factor (NGF) in the flexor carpi ulnaris muscle of the forearm and pressure pain thresholds were collected at day 0 (control), day 7 (peak) and day 14 (recovery). A second cohort (n=18) underwent an identical procedure, however, half received a placebo between days 0-7 before switching to minocycline from days 7-14 (P1/M2), while the remaining subjects received minocycline (day 0: 200mg then 100mg b.i.d. for 7 days) before switching to placebo (M1/P2).

Results: The initial cohort exhibited a diffuse muscular pain hypersensitivity with a decrease in pressure pain thresholds at day 7 before a partial return to normalcy at day 14. The P1/M2 treatment group exhibited an identical peak in hypersensitivity at day 7, however, after switching to minocycline in week 2 showed a significant reduction in muscle hyperalgesia compared to the initial cohort at day 14. The M1/P2 treatment group had significantly less (~43%) hyperalgesia at day 7 compared to the other groups.

Conclusions: The study indicates that the administration of minocycline can reduce experimentally induced muscle pain regardless of the time of administration.

Introduction

Despite growing knowledge of pain mechanisms in humans, the efficacy of current pain treatments remains limited (Moore 2013). Nerve growth factor (NGF) is a naturally occurring neurotrophic factor which shifts from signalling nerve growth and survival during development to signalling inflammation and injury in the adult nervous system (Lewin et al., 2014; Pezet and McMahon 2006) with NGF synthesis in skeletal muscle increasing under pathological conditions (Amano et al., 1991; Hayashi et al., 2011; Murase et al., 2010). The pro-inflammatory role of NGF and the identification of elevated levels of NGF in many conditions such as arthritis (Aloe et al., 1992; Halliday et al., 1998; Raychaudhuri and Raychaudhuri 2009) and fibromyalgia (Giovengo et al., 1999) has implicated NGF as a viable target for the treatment (Hefti et al., 2006; Kumar and Mahal 2012; Watson et al., 2008) and experimental modelling of chronic pain (Andersen et al., 2008; Bergin et al., 2015; Deising et al., 2012; Hayashi et al., 2013; Svensson et al., 2008).

Intramuscular administration of NGF produces a hyperalgesia (Andersen et al., 2008; Gerber et al., 2011; Hayashi et al., 2013; Svensson et al., 2003; Svensson et al., 2008) that can manifest as movement evoked pain and pain to palpation (Bergin et al., 2015). The long-lasting (days to weeks) effects of NGF administration (Nie et al., 2009; Svensson et al., 2003) are thought to stem from the activation of a cascade of neurotrophic factor-mediated changes (Pezet and McMahon 2006; Priestley et al., 2002) including the upregulation of neuromodulators such as calcitonin gene-related protein and substance P (SP) (Malcangio et al., 1997) as well as enhanced responsiveness of ion channels (Pezet and McMahon 2006).

With such widespread effects, NGF has been proposed as an experimental pain model of sustained elbow pain (Bergin et al., 2015), temporomandibular disorder (Svensson et al., 2003), and myofascial (Hayashi et al., 2013) and neuropathic pain (Rukwied et al., 2010).

We have previously shown that in healthy participants minocycline treatment prevents muscle hyperalgesia and cutaneous allodynia induced by repeated intramuscular injections of hypertonic saline (HS) (Samour et al., 2017). Minocycline is a second-generation, semi-synthetic tetracycline, and is widely used as a broad-spectrum antibiotic (Strauss et al., 2007; Yong et al., 2004). Minocycline has been shown to reduce microglial activation (Tikka et al., 2001; Tikka and Koistinaho 2001) and given the identified role of microglia in neuropathic pain (Tsuda 2016), this reduction in microglial activation may cause a

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suppression of the pain response. Importantly, for potential therapeutic applications, minocycline is lipid-soluble and can penetrate the blood-brain barrier allowing it to act on the central nervous system (Brogden et al., 1975; Kielian et al., 2007; Yrjänheikki et al., 1999). Indeed, it has already been reported that minocycline administration in a clinical context can have beneficial effects in humans with improved functional recovery in patients with spinal cord injury (Casha et al., 2012) and a reduction of the neuropathic symptoms in patients with diabetic neuropathy (Syngle et al., 2014).

Based on the above observations, and our prior finding that minocycline prevented the development of muscle hypersensitivity (Samour et al., 2017), we hypothesised that minocycline would limit the development (when given pre-emptively on day 0) and reverse the established (when administration was delayed until day 7) muscle hypersensitivity induced by repeated injections of NGF. By working in a model system that mimics persistent pain hypersensitivity we will be able to evaluate the potential for minocycline to have efficacious translational effects in a clinical cohort within the context of muscle pain. To this point, the effects of minocycline were examined in a double-blind placebo-controlled drug-crossover study with treated individuals additionally compared to an untreated cohort.

Methods

Subjects with no reported history of musculoskeletal or neurological disorders were recruited for this study (18-39 years old; mean age 21.6 ± 0.9 SD). Subjects were asked to refrain from strenuous bouts of physical activity for at least 48 hours prior to commencement of the study so as not to sensitize any muscles, particularly in the forearm (Weerakkody et al., 2001). Informed written consent was obtained from each subject prior to commencement of the study. Across all experimental sessions, the subjects sat comfortably in a chair in a semi-reclined position. The study was approved by the Human Research Ethics Committee (approval number: H9190) of Western Sydney University in accordance with the Revised Declaration of Helsinki of 1975, as revised in 1983.

Figure 1 depicts a timeline of the study for all cohorts tested, indicating NGF injection schedule and sensory testing sessions as well as drug-crossover within cohort 2.

Insert figure 1 here

NGF Injection

Recombinant human β -NGF (5 μ g; Peprotech, Rocky Hill, New Jersey, USA) was dissolved in a solution of 50 μ L of sterile water (Pfizer Australia Pty. Ltd., West Ryde, NSW, Australia). The NGF solution was injected as a bolus into the belly of the *flexor carpi ulnaris* (FCU) muscle of the non-dominant arm at a point approximately 8-9 cm distal to the medial epicondyle of the elbow, adjusted in proportion to the arm length of the subject. Injections of 5 μ g were administered on days 0, 2 and 4 of the study using a BD Ultra-Fine II syringe (BD ANZ, Sydney, Australia). Injections were approximately 48 hours apart and repeated at the same site to minimize the number of needle stick injury sites across the muscle test area (FCU). Before each injection, the tenderness of the test site and the effect of previous NGF injection were determined through palpation of the muscle compartment.

A repeat NGF injection model was chosen as it has been shown to elicit a longer lasting reaction when compared to single injection models (Deising et al., 2012; Hayashi et al., 2013; Svensson et al., 2003) and ensure sustained hypersensitivity at day 14 (Hayashi et al., 2013). The interval between the repeat injections (~48 hours) was chosen to mimic the timeline of our previous minocycline work which used repeated HS injections to evoke hypersensitivity (Samour et al., 2017).

Testing Sites

Testing was performed bilaterally across both FCU muscles due to a prior observation of bilateral muscle hyperalgesia in a longitudinal experimental pain model which involved repeated HS injections (Samour et al., 2017).

With subjects comfortably seated, a 6x10cm grid consisting of 15 points was drawn over the anteromedial/flexor compartment of both forearms overlying the FCU muscle. The 15-point grid was arranged with three columns running medial to lateral and five rows running proximal to distal with 2 cm separating each point (Figure 1). The grid was placed in such a manner that the centre point was the same site as the NGF injection (marked in red in Figure 1 and in bold throughout Figure 2).

Pressure pain threshold (PPT) testing

Mechanical algometry was performed across both test sites (bilateral FCU) on three separate occasions to assess PPTs and the distribution of evoked muscle hyperalgesia from intramuscular NGF injections.

Initially, a force of 30 N for ~3 s was applied at each grid point (n=15) across both test sites using a 1 cm² rubber tip pressure gauge (Pain Test Model FPN 50 Algometer, Wagner Instruments, Greenwich, Connecticut, USA). If a participant reported pain at a point, then a progressively smaller force (in decrements of 5 N) was applied until the subject no longer reported a painful percept. To mitigate the chance of central wind-up (an increase in neuronal excitability observed following repeated stimulation, Herrero, et al., 2000) an inter-stimulus interval of at least 10 s was used and threshold values were confirmed by testing adjacent and remote sites in a pseudo-random order before returning to the test site in question. The upper end of the PPT test range (30 N) was selected so as not to evoke a painful response in more than ~30% of the population and aligns with reports in the literature 30 N is the approximate upper border of the innocuous pressure range (Chesterton et al., 2003). Methods for PPT evaluation are based on those previously reported in the literature (Nagi and Mahns 2013; Samour et al., 2017).

Testing intervals were arrived upon based on data collection from a pilot study, with thresholds collected on day 0 prior to NGF injection (control), day 7 after the first NGF injection where hypersensitivity was established, and 14 days after the first injection where threshold values remained significantly elevated compared to control.

Follow-up testing

Subjects were asked to return for a follow-up testing session at least 28 days after the initial NGF injection to ensure a full alleviation of muscle hyperalgesia and a return to normalcy. For this, an abridged sensory protocol was utilised with muscle palpation and pseudorandomised application of pressure algometry across the test site to ensure that there were no residual effects of NGF injections. As no subjects reported tenderness to palpation or algometry during the abridged protocol, a complete protocol (as outlined above) was not performed at this time point.

Minocycline administration

Based on the standard Australian prescribing practice and previous studies (Curtin et al., 2017; Pachman et al., 2017; Samour et al., 2017), minocycline was given orally as a 200mg loading dose followed by bidaily 100mg continuation doses. Minocycline and placebo (rice flour) tablets were formulated by a compounding chemist in opaque capsules and delivered in coded 'Minocycline trial' containers that were (other than the code) indistinguishable to the subjects and the experimenters.

The code linking the container number to the formulation of its contents was retained by the compounding chemist for the duration of data collection to ensure true double-blinding of the study.

Cohort 1 – Untreated control

Eleven subjects (4 females) were recruited for this arm of the study. These subjects received NGF injections in the absence of a pharmacological or placebo intervention in order to characterise the distribution of evoked hypersensitivity in a repeated intramuscular NGF injection pain model.

Cohort 2 – double-blinded, placebo-controlled minocycline trial

Twenty subjects (7 females) were recruited within this arm of the study. No subjects from cohort 1 were recruited into this arm of the study due to their familiarity with the NGF pain model. The subjects were randomly allocated to a drug treatment group to assess the efficacy of minocycline in ameliorating or preventing NGF induced muscle hyperalgesia.

Participants were randomly allocated (10 in each group) to either receive treatment (minocycline) in week 1 and placebo (rice flour) in week 2 (M1/P2; 8 males, 2 females), or placebo in week 1 and treatment in week 2 (P1/M2; 5 males, 5 females). Subjects took their week 1 loading dose at the conclusion of the first testing and NGF injection session on day 0 and were instructed to start their bidaily dose that evening. The final dose from week 1 was consumed on the morning of day 7, prior to PPT testing on that day, with subjects returning their empty week 1 drug container on this day to ensure compliance. After this, subjects were given their week 2 loading dose and instructed to undertake an identical protocol such that their final dose would be taken on the morning of day 14, prior to final PPT testing and completion of the study.

In order to avoid any expectation of treatment by the subjects, individuals within the drug-crossover study were informed that there were three potential groups they could be placed in, with one of those groups being a placebo-only group which in fact did not exist. This was in addition to being informed of the actual treatment groups in which subjects received minocycline treatment in the first week followed by placebo (M1/P2) or vice versa (P1/M2).

Statistical analysis

Within cohorts, a two-way repeated measures analysis of variance (2-way RM ANOVA) was used to compare the sensory threshold values (PPTs) obtained at days 0, 7 and 14 across the bilateral FCU testing sites. The P1/M2 and M1/P2 treatment groups from cohort 2 were compared with the untreated control group (cohort 1). When significant differences were observed on a group level, Tukey's multiple comparison test was performed to identify individual changes at different applied forces (N) during PPT testing.

D'Agostino and Pearson omnibus test was performed to confirm the presence of normally distributed data. Data are presented as mean $\pm$ standard error of the mean (SEM).

Preliminary power calculations (G Power 3.1) based on the cumulative (maximal) pressure point thresholds using an RM-ANOVA (control, day 7, day 14 and recovery), with two experimental groups (P1/M2 and M1/P2), 9 participants ($\alpha = 0.05$) and a conservative effect size of 0.3 suggested a power of 0.84. Minocycline reduced the NGF induced hypersensitivity by over 40% resulting in an effect size of 0.86 and power >0.99 .

Results

Cohort 1 - Untreated control

Eleven subjects (4 females) were recruited within this arm of the study of which one subject (female) exhibited persistent muscle ache and muscular hypersensitivity covering the entirety of the 15 test sites and extending along the limb following two NGF injections. Consequently, the subject elected to not receive the third NGF injection. The subject undertook sensory testing throughout the duration of the study and a hypersensitivity did persist, however, the subject was excluded from analysis as she did not receive all NGF injections. Acutely, the injection of NGF was non-painful in 7 out of 10 subjects with the other 3 subjects reporting a transient (~ 20 s) sharp pain upon injection. This acute pain was

not present at the first NGF injection and was only reported for the subsequent injections. None of the subjects (except for the one excluded) reported a persistent background pain within the FCU muscle at rest, but rather a pain to palpation or exertion of the muscle and adjacent musculoskeletal structures.

Under control conditions, at day 0, the maximal force of 30 N produced no pain in all except two subjects where a single tender point (out of 15 test sites) was reported in each, resulting in an average of 0.3 ± 0.2 tender points reported across the cohort (Figure 2A).

One week after the start of NGF injections (day 7), muscle hyperalgesia was maximal at the central injection site (marked in bold throughout Figure 2), where all individuals (**100%**) reported a painful response, and was surrounded by test sites where progressively higher forces were required to evoke responses with ~two-thirds of all test sites (**Σ64%**: total number of responses divided by total number of trial sites (15) in 10 subjects = 96/150) evoking a painful response at 30 N.

When the spatial data were replotted as the cumulative number of tender points over the testing range (5-30 N), the use of 30 N resulted in the emergence of 10 ± 0.9 muscle sites as tender, a significant increase from day 0 ($p < 0.0001$). The minimal threshold values ranged between 5-15 N (mean 8.5 ± 1.4 N) and were associated with a significantly ($p < 0.0001$) increased number of responses between 5 and 30 N, indicating that the fall in threshold was accompanied by an increased sensitivity (left shift) in the subjects' pain response profile.

Consistent with ongoing recovery, the number of tender points at day 14 had fallen to **Σ33%** of all tested sites with reduced responses at 20, 25 and 30 N (3.3 ± 0.9 , $p = 0.0066$; 4.7 ± 1.2 , $p < 0.0001$; 5.7 ± 1.4 , $p < 0.0001$, respectively). The majority of subjects (80%) reported minimum thresholds within the range of 5-20 N (12.9 ± 2.4 N), a value which was significantly elevated from the minimum thresholds obtained at day 7 ($p = 0.0118$) yet was still significantly less than baseline (day 0) values ($p < 0.0001$).

Follow up testing at least 28 days post the initial NGF injection revealed a complete return to normalcy in all subjects.

No changes were observed in the contralateral FCU following repeat NGF injection with 30 N force eliciting an average of 0.2 ± 0.2 tender points at day 0, 0.3 ± 0.2 at day 7 and no tender points at day 14 ($p = 0.2356$). Given this constraint, only the effects of NGF injection

within the ipsilateral limb will be discussed in further cohorts. The effects observed in the ipsilateral limb were not solely the result of wind-up phenomena associated with repeated testing as no change was observed when the same testing was used in the contralateral limb.

Insert Figure 2 here

Cohort 2- double-blinded placebo-minocycline cross-over trial

In total 20 subjects were recruited as part of this cohort, with subjects randomly allocated to either treatment group (n=10 per group). Of the subjects randomly allocated into the P1/M2 group, one subject (female) reported nausea and intestinal discomfort during the minocycline treatment phase and withdrew from the study. To account for an uneven cohort between the two treatment groups, a random number generator was used to exclude an individual (male) from the M1/P2 treatment group to allow for a comparative *n* across both groups within this cohort (results n=18). Sequential analysis based on a subject-by-subject exclusion of one participant from this group did not alter the significance of these findings between groups.

Subjects within this cohort reported similar symptomology in response to NGF injection as cohort 1 with acute NGF injection remaining painless in the majority of subjects with a lack of tonic muscle pain in the injected region for the study duration, but rather a pain to exertion or palpation.

P1/M2 PPT examination

In those receiving the placebo first (P1), mapping the spatial extent of hypersensitivity revealed a pattern indistinguishable from that observed in the untreated group (cohort 1) at day 7 that was characterised by a high sensitivity (average minimum of 9.4 ± 2.6 N; range 5-25 N) at the injection site where all subjects (100%) reported pain, surrounded by sites of decreasing sensitivity and responsiveness (Figure 2B). Overall **~564%** of all test sites (86/135 total sites) displayed a response at ≤ 30 N. A two-way RM ANOVA of the cumulative number of tender points indicated that placebo had no effect on the spatial extent or intensity of muscle hypersensitivity at day 7 compared to the untreated cohort with 10 ± 0.6 points being reported as tender at ≤ 30 N ($p < 0.0001$) and elevated response levels at forces of 10-

30 N ($p < 0.005$). When the same participants received minocycline in the second week (M2), fewer individuals responded (67%) at the central injection site (Figure 2B) with the number and sensitivity of responses at adjacent test sites declining more rapidly than that observed in their day 7 responses or those observed in the untreated week 2 groups (Figure 2A). Overall the number of responsive sites was reduced to **~22%** or 3.1 ± 0.9 points at ≤ 30 N, significantly fewer than those reported at the same time point in the untreated cohort (5.7 ± 1.4 responses at 30 N, $p < 0.05$).

Additionally, a significant reduction was observed between tender points observed at day 7 and 14 for forces of 10 N and greater ($p < 0.05$), suggesting a functional recovery. The number of tender points at 30N observed at baseline (day 0) and at day 14 were not significantly different ($p = 0.0791$).

M1/P2 PPT examination

In subjects who received minocycline treatment in week 1 (and placebo in week 2, M1/P2) a significant interaction ($p < 0.0001$) between the observed number of tender points at varying forces across the testing period (Figure 3) and treatment group was observed. At day 7 only 67% of subjects displayed threshold values in the testing range (mean 11.7 ± 3.4 N; range 5-20 N) and the spatial extent of the hypersensitivity was confined to **36%** (48/135) of all the tested sites (Figure 2C). Likewise, the cumulative data revealed significantly fewer tender points (5.7 ± 1.7 at ≤ 30 N) compared to untreated ($p = 0.0019$) and placebo-controlled groups ($p = 0.0053$) at this time point. The 5.7 ± 1.7 tender points reported to 30 N of force application represent an approximately **43%** decrease in reported cumulative tender points compared to both cohort 1 and the P1/M2 treatment group at this time point which has been shown to be the peak of hypersensitivity. Measurements across all 15 sites at forces of 5 N and 10 N showed no significant differences ($p > 0.05$) in week 1 when compared to baseline (day 0).

Contrastingly, comparisons between the PPT values obtained at day 14 and baseline show no significant variation ($p > 0.05$) across all applied force levels. However, significant variation was seen between day 7 and 14 measurements at 25 N ($p = 0.0225$) and 30 N ($p = 0.0036$).

Insert Figure 3 here

Day 14 data as a function of peak sensitivity (day 7)

When data obtained at day 14 are represented as a function of the peak sensitivity reported at day 7, a significant difference is observed between the M1/P2 and P1/M2 treatment groups in comparison to the untreated controls. The P1/M2 treatment had a significantly increased amelioration of muscle tender points by day 14 as compared to the M1/P2 group ($p=0.0003$). This is evident at 30 N with the P1/M2 only reporting $37 \pm 8\%$ of the tender points reported at day 7 as still being tender at day 14 while the M1/P2 group still reported $60 \pm 11\%$ of the day 7 tender points as painful at day 14 testing. The response of the M1/P2 group at day 14 is similar to the response of the untreated control cohort which reported $62 \pm 11\%$ of the day 7 tender points as still painful at day 14. Thus, despite minocycline significantly reducing overall tender points at day 7 in the M1/P2 treatment group, the switch to placebo in the second week of the study results in the same subsequent recovery from peak hypersensitivity as a lack of treatment.

Discussion

In this study we characterised the sensory effects of repeated intramuscular NGF injections in healthy subjects, revealing a robust muscle hypersensitivity (hyperalgesia) that extended to all 15 test sites overlying the FCU (NGF affected muscle). This NGF-induced hypersensitivity did not extend to contralateral test sites, in contrast to observations following repeated HS injections (Samour et al., 2017). In the current placebo-controlled double-blind drug-crossover trial, minocycline was found to significantly reduce the onset of muscle hyperalgesia when given concurrently with NGF injection (M1/P2) and was equally able to alleviate muscular hypersensitivity when administered after peak muscle hyperalgesia (P1/M2). This extends our previous finding that minocycline ameliorates the development of muscle hyperalgesia when administered concurrent to the induction of HS-induced muscular pain hypersensitivity (Samour et al., 2017).

Progressive muscular hyperalgesia was observed in the NGF injection site with significantly decreased PPTs in all subjects regardless of the timing of minocycline intervention. This mechanical hyperalgesia manifested as an increase in the distribution and number of tender points across the affected FCU muscle and in adjacent muscles, peaking at day 7 and persisting through to the week 2 testing session. The hyperalgesia spread across the entirety

of the compartment, despite the use of a single injection site. A somatopically diffuse region of pain hypersensitivity evoked by bolus intramuscular NGF injection has been reported before in the context of contraction-induced muscle pain (Sorensen et al., 2018). The finding of a spatially diffuse distribution of hypersensitivity from repeated bolus NGF injection suggests that this model could be used in future experimental studies of prolonged muscle pain and hyperalgesia.

The finding of muscle hyperalgesia mimics the diffuse muscle pain hypersensitivity commonly observed in chronic musculoskeletal pain conditions such as lateral epicondylitis (tennis elbow), temporomandibular joint disorders and fibromyalgia (Andersen et al., 2008; Bergin et al., 2015; Gerber et al., 2011; Hayashi et al., 2013; Svensson et al., 2003). Indeed, the lack of contralateral interactions observed in the NGF pain model has also been described previously (Svensson et al., 2008), however, this was in response to a single intramuscular injection of 5 µg of NGF. This does provide a point of contrast to the HS pain model where bilateral interactions have been shown in an experimental design which had an identical time course to this study (Samour et al., 2017). A key difference between the two experimental models may be the inherently painful nature of HS injection which consistently is able to evoke an acutely painful percept, as opposed to the largely innocuous nature of acute NGF injection with only a minority of subjects reporting acute pain, however those subjects still exhibited no bilateral tenderness. Reports of acute pain from intramuscular NGF injection are varied with only a minority of papers reporting an acute sharp pain at the time of injection (Gerber et al., 2011; Hayashi et al., 2013). The lack of acute pain in other reported literature could be due to the single injection protocol which is favoured in the literature (Andersen et al., 2008; Bergin et al., 2015; Deising et al., 2012; Svensson et al., 2003; Svensson et al., 2008). It is possible the transient pain reported in some subjects is a result of the NGF vehicle with sterile water for BP typically having a pH between 5.5 and 7.5. Whilst intramuscular injection of acidic solutions is known to result in pain (Frey Law et al., 2008; Jensen and Norup 1992), the small injection volume used in this study (50 µL) and the absence of acute pain following the first NGF injection indicates that this pain is more likely resultant from NGF evoked sensitisation. Additionally, pain was only elicited on repeat injections, with the primary injection of NGF remaining innocuous, further indicating that acute pain within this study is likely resultant from the progressive

sensitisation of the NGF-injected region. Whether the presence of overt pain at the time of injection favours the development of a more profound and extensive pattern of pain hypersensitivity across limbs remains untested.

The most intriguing finding of this study is the ability of minocycline to significantly alleviate the muscle hyperalgesia associated with intramuscular NGF injections. This alleviation appears to be independent of the time of onset of mechanical hypersensitivity as beneficial effects were observed for the onset (M1/P2) and established (P1/M2) treatment groups. In subjects who received minocycline in the first week (M1/P2) there was a significant decrease in maximum tender points elicited at 30 N when tested on day 7 at peak hypersensitivity (Figure 2). There was an approximate reduction in the cumulative number of reported tender points of **43%** in this treatment group compared to the untreated cohort and the group which received placebo during week 1 (P1/M2). Accordingly, at this time point a reduced cumulative number of tender points correlated with a lesser degree of somatotopic spread across the anterior forearm (Figure 1). Additionally, in the P1/M2 treatment group, there was an enhanced amelioration of hypersensitivity between days 7 and 14 when compared to the other treatment groups who received either placebo or no treatment within this time period. Minocycline treatment led to **20-25%** fewer cumulative tender points at day 14 as a function of the tender points at day 7, demonstrating a clear efficacious effect in the recovery of an already established muscular pain hypersensitivity.

Critical to the above findings, when subjects received a placebo during the induction of NGF-evoked hypersensitivity (P1/M2) there were no differences in the first week from untreated controls, indicating a lack of placebo effect within this group. Similarly, cessation of treatment within a group who received minocycline for week 1 (M1/P2) resulted in identical recovery between weeks 1 and 2 as untreated controls, further indicating a lack of placebo effect across the study. We believe the reason for the absence of a placebo effect may be related to the study design, in particular how the expectations of the subjects were managed. In contrast to clinical trials (where the logical expectation is that treatment will alleviate existing pain, i.e., meeting the Latin derivation of placebo: *I will please*), this study used healthy subjects (cohort 2) that were naïve as to the impact of repeat NGF injections and the possible impact of minocycline (or indeed which agent, NGF or minocycline, was the stimulus and which was the treatment). The healthy subjects' sole task was to report which

of the 15 PPT test sites were painful during each testing session. The subjects were informed that study design was a cross-over design with an equal likelihood of receiving placebo in the first week, second week or in both weeks. Previous studies examining the effects of minocycline on muscle hyperalgesia have reported a substantial placebo effect (~35%), however, that may be due to the two-arm design of the study as subjects were informed that there were only two treatment allocations, minocycline or placebo (Samour et al., 2017). The use of a drug-crossover design in this study led to a lack of observable placebo effect and thus our findings suggest that minocycline treatment has an efficacious effect on muscle hyperalgesia.

The exact mechanism by which minocycline is exerting this effect is beyond the scope of our study, however, based on the available literature regarding minocycline and NGF, several interactions may be occurring. The glial suppressive effects of minocycline have been widely reported (Amin et al., 1996; Tikka et al., 2001; Tikka and Koistinaho 2001) and equally, it has been demonstrated that microglial activation is implicated in the cascade initiated by peripheral NGF administration (De Simone et al., 2007; Pezet and McMahon 2006). Indeed it has been concluded in multiple animal studies that the efficacious effects of minocycline on experimentally induced pain is the result of a corresponding decrease in microglial activation (Chew et al., 2014; Hua et al., 2005; Ledeboer et al., 2005; Raghavendra et al., 2003) and synaptic relay in the substantia gelatinosa (Huang et al., 2014; Peng et al., 2016). Minocycline has also been shown to suppress the interaction of primary afferent neurons with stellate cells (Afroz et al., 2019) and macrophages (Dutta et al., 2010) in the periphery. Whether the effect of minocycline in humans is the result of central or peripheral effects, or likely a combination of the two, warrants further investigation.

NGF has been shown to directly upregulate neurotrophic factors including SP (Lewin et al., 2014; Minnone et al., 2017; Pezet and McMahon 2006) which has long been regarded as necessary for the development of central sensitisation (Khasabov et al., 2002; Malcangio et al., 1997; Xu et al., 1992). The involvement of SP in the formation of the centralized effects of NGF was shown in isolated spinal cords where antagonism of SP significantly reduced NGF evoked responses (Thompson et al., 1995). Increased SP expression as a result of NGF could result in the activation of microglia which would further release inflammatory markers and thus contribute to central sensitisation (Kreutzberg 1996; Watkins et al., 2001).

Additionally, retrograde transport of NGF along sensory terminals to trk-A positive neurons has been shown to activate the glutamatergic system with the phosphorylation of NMDA subunits (Pezet and McMahon 2006; Svensson et al., 2008). Furthermore, NMDA receptors in the periphery are known to modulate muscle nociceptor activity (Wong et al., 2014).

More broadly, aberrant glial cell activation has been implicated in the development of chronic pain conditions (Bettoni et al., 2008; Jha et al., 2012; Loggia et al., 2015; Nijs et al., 2017; Scholz and Woolf 2007). As such, the anti-microglial properties of minocycline have already been examined in a range of conditions including Parkinson's (Wu et al., 2002), multiple sclerosis (Popovic et al., 2002), spinal cord injury (Stirling et al., 2004) and Alzheimer's disease (Garcez et al., 2017; Seabrook et al., 2006). In animal models, minocycline has been shown to significantly reduce mechanical hyperalgesia (Boyette-Davis and Dougherty 2011; Boyette-Davis et al., 2011; Hua et al., 2005; Ledebroer et al., 2005; Morgado et al., 2011; Raghavendra et al., 2003). This efficacious effect of minocycline in mechanical hypersensitivity has been somewhat less convincing in human studies with the beneficial effects reported in conditions including rheumatoid arthritis (Langevitz et al., 2000), spinal cord injury (Casha et al., 2012) and diabetic neuropathy (Syngle et al., 2014) matched by reports of non-efficacious or even detrimental effects in a range of other conditions (Arout et al., 2018; Curtin et al., 2017; Pachman et al., 2017; Sumitani et al., 2016; Vanelderen et al., 2015). Intriguingly the lack of efficacy in pain alleviation may come down to study design with many using qualitative measures in the absence of acute stimuli (Curtin et al., 2017; Pachman et al., 2017; Sumitani et al., 2016; Vanelderen et al., 2015) whilst others use supramaximal stimuli (Arout et al., 2018) that are and should reasonably remain painful; in contrast those used in the current study traversed a range of intensities that were innocuous under control conditions but became painful following repeated NGF injections.

The sex imbalance within this study stands as a potential limitation with only approximately a third of the study participants being female. This imbalance is an important consideration due to a higher prevalence of chronic pain in females (Bartley and Fillingim 2013). However, more pertinent to the findings of the current study is whether sex has a role in the efficacy of minocycline for the treatment of pain. There are conflicting reports on this in the mouse literature with initial evidence suggesting that how effective minocycline was on alleviating

pain were sex-independent (Bastos et al., 2013), though, more recent reports have concluded that minocycline is more efficacious in males (Chen et al., 2018; Fernandez-Zafra et al., 2019). However, such observations in humans remain untested, necessitating further investigation if minocycline is to be considered for the alleviation of clinical pain.

Conclusions

Minocycline significantly reduced the muscle hyperalgesia evoked by NGF when administered concurrently with NGF injections and pain hypersensitivity development. Additionally, when minocycline was administered following the establishment of muscle hyperalgesia, it was able to significantly and efficaciously facilitate recovery compared to untreated and placebo counterparts. The data obtained in this study demonstrate that giving minocycline pre-emptively or following the onset of muscle tenderness reduces muscle hyperalgesia.

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Author Contributions

SSN and DAM made substantial contributions to the study design and conception. JSD was responsible for data acquisition and analysis as well as drafting the original manuscript. The manuscript was subsequently revised critically for important intellectual content by SSN and DAM before all authors approved the final manuscript before submission for publication.

Legends

Figure 1. *Study design across the untreated and double-blind placebo-controlled minocycline crossover with injection and PPT test sites.* A timeline depicting NGF injections and PPT testing sessions across the two-week study duration. In the placebo-controlled, cross-over study participants were randomly assigned to start (day 0) with placebo (P1/M2) or minocycline (M1/P2) and switch to minocycline/placebo, respectively on day 7. PPTs were measured before (days 0 and 7) subjects took a 200mg loading dose (grey) and provided with continuation doses (100mg bidaily for 7 days, green). Final PPT testing was conducted

on day 14. The test sites (black dots) and NGF injection site (red dot) are shown in the lower figurine.

Figure 2. *Spatial distribution (% of subjects with a threshold $\leq 30\text{N}$) and average pressure pain thresholds (PPT, coloured scale) prior to (day 0, control), 7 and 14 days after the start of repeat NGF injections, and their improvement following minocycline treatment. Average PPTs are coded from red (5 N) to white (30 N). Each square corresponds to a test site overlying the FCU muscle as shown in Figure 1. Sites where no PPTs were recorded within our test range (i.e. PPT $>30\text{N}$) are marked with an X. **A. Untreated** subjects (n=10): 7 days after the first NGF injection, mechanical hyperalgesia focused on the central injection site (marked in bold) was reported by all (**100%**) and extended to ~two-thirds (**Σ64%**) of sites tested (10 subjects x 15 sites: 96/150); **14 days** following repeat NGF, 67% of subjects reported hyperalgesia that was confined to **Σ33%** of all sites tested. **B.** Subjects treated with placebo first (**P1/M2**, n=9) were indistinguishable from untreated subjects at day 7 but showed enhanced recovery (elevated thresholds and fewer tender points, **Σ22%**) following minocycline treatment at day 14. **C.** Subjects treated with minocycline first (**M1/P2**, n=9) developed a reduced hypersensitivity (at day 7) that was comparable to that observed in the recovery (day 14) of untreated and P1/M2 groups.*

Figure 3. *Muscle tender points (mean $\pm$ SEM) reported across an NGF-injected FCU in control and minocycline treated cohorts. The untreated control group (cohort 1) received no treatment (n=10). In the treatment groups, one group (n=9) received minocycline until day 7 before receiving placebo in week 2 (M1/P2) while the other (n=9) received placebo followed by minocycline (P1/M2). The day 0 recordings were obtained prior to NGF injection and show no statistical variation between groups. The statistical variance was observed between the untreated control group and the M1/P2 group at the end of week 1 and persisted until week 2 at higher forces. No placebo effect was observed at week 1 in the P1/M2 group, however, retrospective minocycline treatment resulted in significantly reduced tenderness at 30 N in this group. Total n = 28.*

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Untreated Control

Double blinded, placebo-controlled, cross-over trial