Influence of Intramuscular Nerve Growth Factor Injection on the Response Properties of Rat Masseter Muscle Afferent Fibers

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Aims: *To investigate whether local administration of nerve growth factor (NGF) decreases the mechanical threshold (MT) of putative nociceptive masseter afferent fibers as part of its mechanism of mechanical sensitization.* ***Methods:*** *Electrophysiologic recordings were made from masseter afferents and a randomized, blinded approach was used to test the effects of intramuscular injection of NGF (2.5 or 25 μg/mL) into the rat masseter muscle on the MT of masseter afferents (n = 65) and plasma protein extravasation.* ***Results:*** *The plasma protein extravasation data and electrophysiological recordings indicated that rat NGF injection was not inflammatory and did not evoke afferent discharge or induce mechanical sensitization (as reflected in a decreased MT) in masseter afferents in either male or female rats. To investigate whether the lack of effect of NGF injection might be due to differences between human and rat NGF, additional experiments with human NGF injection (25 μg/mL) were undertaken. Intramuscular injection of human NGF into the rat masseter muscle also failed to evoke afferent discharges; however, it did decrease the MT of masseter afferent fibers.* ***Conclusion:*** *The finding that neither rat nor human NGF excited putative nociceptive masseter afferent fibers is consistent with a previous report that intramuscular NGF injections are not acutely painful in human subjects. The ability of human NGF injection into the rat masseter muscle to induce afferent mechanical sensitization suggests that this experimental approach may be useful for the study of peripheral mechanisms of myofascial pain and tenderness associated with temporomandibular disorders.* J OROFAC PAIN 2006;20:325–336

Key words: afferent fibers, masseter muscle, muscle pain, nerve growth fibers, temporomandibular disorders

Temporomandibular disorders (TMD) are the most common chronic craniofacial pain condition.[1] TMD are characterized by pain in the temporomandibular joints (TMJ) and/or muscles of mastication, such as the masseter muscle.[2,3] TMD are more prevalent among women than men,[4] and this difference is thought to be partly due to biological factors, such as the sex hormone estrogen.[5] Indeed, craniofacial pain sensitivity appears to vary across the menstrual cycle.[6,7] It has been speculated that local elevation of nerve growth factor (NGF) may be another biological factor that could contribute to TMD-related pain.[8] This idea has been bolstered by the finding that intramuscular injection of human NGF (0.1

μg/kg) into the masseter muscles of healthy men resulted in a prolonged localized mechanical sensitization that was associated with pain during activities involving jaw movements, such as chewing and yawning.[9] Thus, human NGF injection in the masseter muscle produces symptoms similar to those experienced by myofascial TMD sufferers; however, the mechanism that underlies this effect is not yet understood.

NGF is a neurotrophic protein that exerts its biological effects by acting on 2 different receptors: tyrosine kinase receptor A (tropomyosin-related kinase A [TrkA]) and p75 receptor.[10] It is thought that in the peripheral tissues, NGF is responsible for maintaining the sensitivity of primary afferent fibers and that upregulation of NGF can result in alterations in pain-related behavior. Animal studies have shown that exogenous NGF administration leads to hyperalgesia and sensitization of spinal cord dorsal horn nociceptive neurons.[11–15] In adult rats, systemic administration of NGF results in an early phase of thermal sensitization starting 30 minutes after injection and a late but longer-lasting phase of mechanical sensitization starting several hours after NGF injection.[16,17]

The purpose of the present study was to investigate whether local administration of NGF decreases the mechanical threshold (MT) of masseter afferent fibers as part of its mechanism of mechanical sensitization. As it is not feasible to record masseter afferent fibers in human subjects, an acute, anesthetized rat model was used to assess NGF-induced changes in the excitability of individual masseter afferents.[18] Plasma protein extravasation into the masseter muscle after NGF injection was also assessed to determine whether NGF causes local tissue inflammation. It was hypothesized that an intramuscular injection of NGF would cause a decrease in the MT of primary afferents in the masseter muscle in a sex-dependent manner (females showing a greater degree and duration of sensitization than males). Since no gross signs of muscle inflammation were observed after injection of NGF into the human masseter muscle,[9] it was hypothesized that the injection of NGF would not induce muscle inflammation.

Materials and Methods

Surgical Procedures

A total of 65 adult Sprague-Dawley rats (33 males and 32 females) were used for rat NGF experiments. In vivo single-unit recordings were conducted using electrophysiological recording techniques; these techniques and the experimental setup have been described previously.[18] Briefly, for each experiment a rat was weighed and then anesthetized with isoflurane. Following tracheotomy, the rat was mechanically ventilated with a mixture of isoflurane (2% to 2.5%) and oxygen (98% to 97.5%) at a steady rate. A rectal temperature probe was used to monitor the core body temperature of the rat. Electrocardiogram leads were used to monitor the heart rate of the animal. In female rats, a vaginal lavage was performed and a sample of epidermal cells was collected. These samples were examined on a glass slide with a microscope to determine the estrus stage. The femoral artery was catheterized to monitor the mean arterial blood pressure of the rat throughout the experiment. The blood pressure was maintained above 60 mm Hg throughout the experiment and during recordings. The mechanical ventilator rate and isoflurane levels were adjusted accordingly to keep the blood pressure between 60 to 80 mm Hg and end-tidal CO_2 between 2.5% to 6.25%. The femoral vein was also catheterized to administer fluids and Evans' blue dye and to deliver pentobarbital at the end of the experiment to sacrifice the rat. The rat's head was positioned in a stereotaxic frame, and a trephination was made on the right side of the skull so that a recording electrode could be lowered into the right trigeminal ganglion. A blunt dissection was conducted to expose the caudal brainstem to permit electrical stimulation of central endings of ipsilateral masseter afferent fibers with a stimulating electrode.

Recording Procedures

A parylene-coated tungsten recording electrode (2 MΩ, A-M Systems) was lowered into the trigeminal ganglion to record from the cell body of a single primary afferent. The output from the recording electrode was fed into a computer (Spike 2 software, Cambridge Electronic Design).[18] A blunt mechanical search stimulus was used to find and activate a primary afferent fiber in the masseter muscle. Then the skin overlying the afferent's mechanoreceptive field was pulled away from the muscle and pinch and pressure stimuli were applied to the skin to confirm that the putative nociceptive field of the fiber was in the masseter muscle. Also, the mandible was moved up, down, and laterally to test whether the fiber fired in response to jaw movement and to confirm that the fiber was a masseter muscle afferent and not a joint afferent. Previous anatomical studies have

indicated that putative masseter muscle nociceptors project to the ipsilateral subnucleus caudalis of the trigeminal sensory nuclear complex.[19–21] To confirm the projection of the primary afferent fiber to the subnucleus caudalis, a stimulating electrode was lowered into the caudal brainstem to produce an antidromically conducted action potential. The projection of the fiber to the brainstem was confirmed by collision of orthodromic action potentials evoked by mechanically stimulating the mechanoreceptive field of the fiber in the masseter muscle through the overlying facial skin with antidromic action potentials (generated by directly stimulating the caudal brainstem).

The baseline MT (minimum force required to evoke a primary afferent fiber response) of the fiber was recorded with an electronic von Frey hair (VF hair; blunt polypropylene tip, diameter 0.5 mm, Model 1601C, IITC) every minute for 10 minutes. The jaw was opened to determine if the primary afferent fiber responded to jaw opening, and the distance at which the fiber responded was recorded and termed the effective jaw-opening distance. For the fibers that did not respond to jaw opening, the maximal jaw-opening distance was recorded.

Experimental Protocol

Prior to the beginning of the NGF study, 2 preliminary experiments were conducted in female rats with 1 mol/L glutamate solution (10 μL injection, pH 7.4, Sigma Chemical, St Louis, MO). These experiments were done to test the sensitivity of the technique to detect MT changes over a prolonged period of time. Glutamate is already known to evoke significant masseter afferent fiber discharge and to induce a period of mechanical sensitization.[18]

For the NGF study, each primary afferent fiber was randomly assigned to 1 of the following 3 groups: vehicle control (10 μL of phosphate-buffered isotonic saline and albumin, n = 20), 2.5 μg/mL rat NGF (~0.1 μg/kg, 10 μL, n = 20; Sigma Chemical), and 25 μg/mL rat NGF (~1 μg/kg, 10 μL, n = 20; Sigma Chemical) dissolved in phosphate-buffered isotonic saline and albumin. The concentration of rat NGF was matched to the dose and concentration of human NGF used in human experiments.[9] All solutions were at physiological pH (pH 7.3 ± 0.1). The investigator conducting the MT recordings was blinded to the injected solution. A catheter (a 26-gauge needle connected to a Hamilton syringe with polyethylene tubing) was used to inject the solution into the mechanoreceptive field of the fiber. After inserting the needle into the muscle, a 10-minute baseline recording was taken to assess any spontaneous firing activity before the injection. At 10 minutes, the solution was injected, and the evoked response was recorded for 10 minutes. Sixty minutes after the injection, 10 consecutive MT recordings were conducted at 1-minute intervals. At the end of the 10-minute recording period, the jaw was opened to determine whether the primary afferent fiber responded to jaw opening and the fiber distance at which it responded. The response to jaw-opening and MT were assessed every hour for a total of 6 hours.

At the end of the recordings, a blood sample was collected to measure plasma estrogen levels with a commercially available radioimmunoassay kit. After blood sample collection, Evans' blue dye (6 mg/kg) was injected into the left femoral vein (via the venous catheter), and the catheter was flushed with normal saline. After 15 to 20 minutes, the rat was euthanized with an overdose of pentobarbital (100 mg/kg) and immediately perfused with 120 mL of normal saline. Following saline infusion, the part of the masseter muscle around the injection site was isolated, weighed, and stored in the freezer for Evans' blue dye analysis. At the end of each experiment, the distance between the recording and the stimulating electrode was measured and divided by the latency of the antidromic action potential to estimate the central conduction velocity of the primary afferent fiber.

All experiments were approved by the UBC Animal Care Committee and adhered to the guidelines of the Canadian Council on Animal Care.

Evans' Blue Dye Absorbance Analysis

Each muscle tissue sample was placed in a test tube and immersed in 2 mL of formamide. The test tube was placed in a water bath at 60°C for 24 hours.[22] The amount of dye extracted from the muscle tissue sample was determined by measuring the absorbance of the supernatant at 620 nm using a spectrophotometer (8452A Diode Array Spectrophotometer).[22] The Evans' blue dye concentration in each muscle sample (μg/g) was calculated.

17β-Estradiol Radioimmunoassay Protocol

Plasma estrogen (17β-estradiol) levels were measured with a commercially available kit (ImmuChem Double Antibody 17β-Estradiol ^{125}I RIA Kit). A radioimmunoassay was conducted to determine the plasma concentration of the unconjugated form of estradiol according to the protocol provided by the supplier (ICN Biomedical).

Data Analysis

Evidence in human subjects suggests that mechanical sensitization of the masseter muscle may occur within 3 hours of NGF injection (Svensson et al, unpublished data). Therefore, data analysis was conducted on MT values recorded every hour for the first 3 hours postinjection. The 10 MT values for each time point were averaged. In order to account for interfiber variability (ie, differences in baseline MT) and compare different populations of fibers, the mean MT for each time point was divided by the baseline mean MT of each fiber to calculate the relative MT. The evoked response of each fiber was calculated by subtracting the total number of the spikes during the 10-minute period before the injection from the total number of spikes during the 10-minute period after the injection. The evoked response of fibers in each group (vehicle control and 2 treatment groups) was averaged to calculate the mean evoked response.

Sample Size

Based on previous human results, it was decided that a difference of at least 25% between the mean mechanical threshold of control and treatment groups was required for the results to be considered biologically meaningful.[23] It was calculated (using a 1-way Analysis of Variance, ANOVA, sample size estimation, SigmaStat 3.0 software, $\alpha = 0.05$, power = 0.80) that a minimum sample size of 10 was required in each group to see a difference of 25% (SD 18%) between the means of the 3 groups. Therefore, a total of 60 individual afferent fibers (n = 60 rats) were examined: 10 males and 10 females for each of the 3 solutions.

Statistical Analysis

A general linear model (GLM) repeated measures 3-way analysis of variance (ANOVA) with covariates of baseline MT, conduction velocity, estrogen levels, and Evans' blue dye levels was used to assess treatment, sex, and time (repeated) effects on the relative mechanical threshold. A 1-way ANOVA was conducted to determine estrus-stage effects on baseline MT and any treatment effect on the evoked response of fibers. The effect of treatment and sex on the Evans' blue dye concentra-tion was analyzed with a 2-way ANOVA. Student *t* tests were conducted to determine sex differences in body weight, plasma estrogen levels, baseline MT, and conduction velocity. Paired *t* tests were conducted to determine whether the blood pressure changed in response to the NGF injections.

A log transformation was carried out to normalize the distribution of baseline MT data, and a square-root transformation was conducted on the Evans' blue dye concentration data. An inverse transformation of rat body weight, a square root transformation of estrogen levels, and a log transformation of conduction velocity were required to create a normal distribution of the data for assessment of sex differences.

A log-linear regression analysis was used to determine the relationship between conduction velocity and baseline MT as well as between plasma estrogen levels and baseline MT for slow (conduction velocity $\leq$ 10 m/s) Aδ fibers.

The level of significance of all statistical tests was set at $P \leq .05$. All values are reported as mean ± SE.

Human NGF Experiments

The majority of previous studies to investigate the effects of NGF on rats have actually employed either human or mouse NGF.[13,16,24,25] Therefore, additional experiments were conducted on 7 female Sprague-Dawley rats with 25 μg/mL human NGF (1 μg/kg, 10 μL; Sigma Chemical) to determine whether there were any differences in the effects of human and rat NGF. The relative MT prior to and for the first 3 hours after NGF injection was calculated as described previously. A repeated measures 1-way ANOVA was used to determine the effect of time on the relative MT postinjection and a post-hoc Holm-Sidak method was used for further comparisons.

Results

To establish that it was possible to conduct primary afferent fiber recordings over long time periods, 2 initial experiments were conducted with glutamate. Recordings of masseter afferent fibers were stable for at least 4 hours postglutamate injection. The glutamate injection into the afferent mechanoreceptive field evoked afferent activity that lasted for approximately 5 minutes (Fig 1 inset). MT decreased postinjection as compared to the baseline (Fig 1a). Also, the fiber became responsive to jaw opening 1 hour postinjection (Fig 1b).

For the NGF study, a total of 71 masseter muscle afferent fibers were recorded from 65 rats, and baseline properties of the fibers, which included conduction velocity, mechanical threshold, and response to jaw opening, were collected. The vast majority of

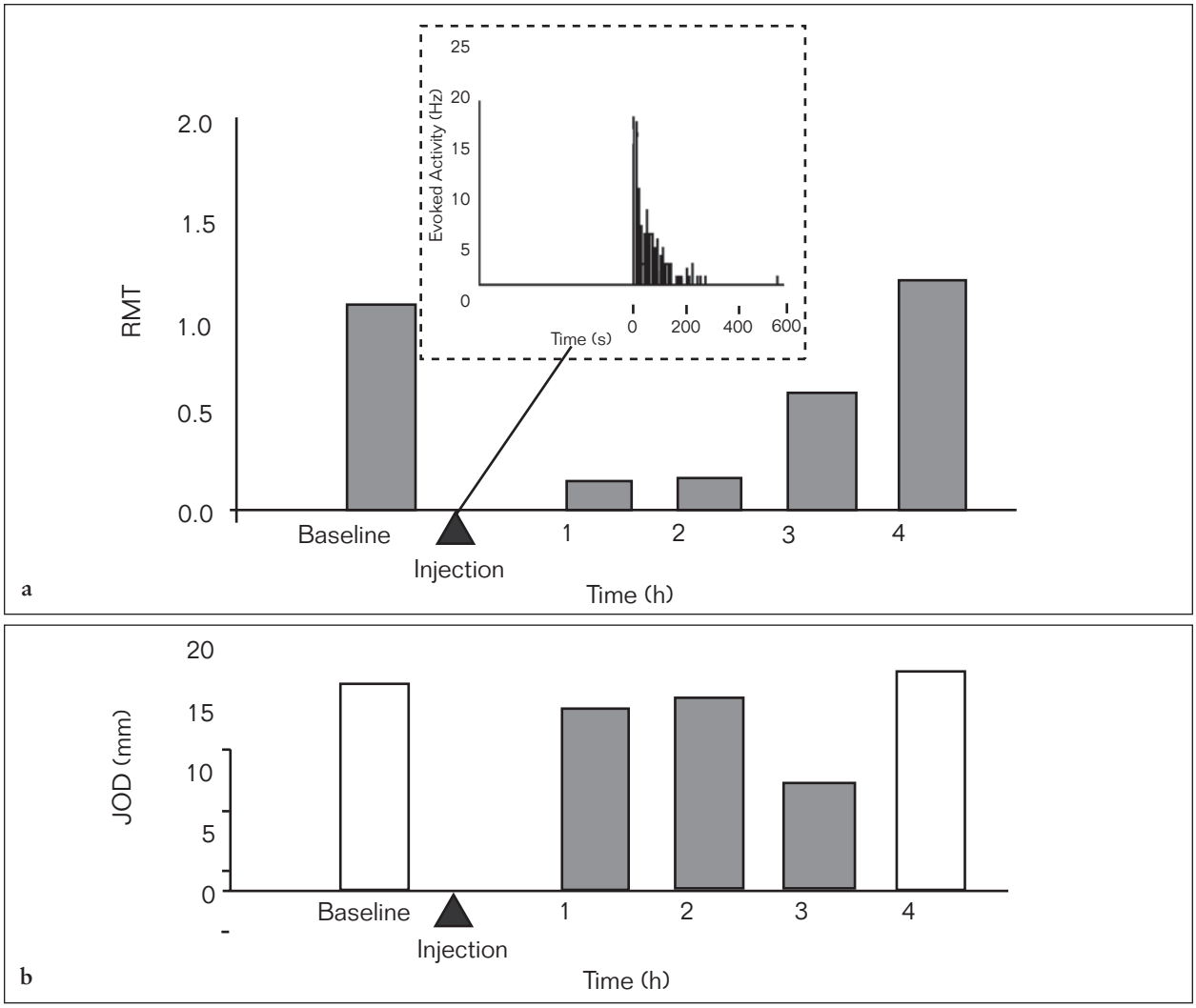

Fig 1 *(a)* Relative mechanical threshold (RMT) of an afferent fiber during baseline and at various time points postinjection. The inset is a histogram of the baseline activity during the 10-minute period before injection and the evoked activity of the fiber in response to the glutamate injection. Note that there was a burst discharge of action potentials. This was typical of responses to glutamate injection. *(b)* Jaw-opening distance (JOD) over time after a 10-μL injection of 1 mol/L glutamate into the masseter muscle. This Aδ fiber (conduction velocity = 2.53 m/s), recorded in a female rat, was unresponsive to jaw-opening before the injection (white bars indicate the maximum distance the jaw was opened) but began firing in response to jaw opening 1 hour after injection. The white bars indicate instances in which the Aδ fiber did not respond; the dark bars indicate the effective JOD for activating the afferent.

these fibers had conduction velocities in the Aδ range (2 to 30 m/s); however, recordings from 3 C-fiber afferents (conduction velocities ≤ 2.0 m/s; 4.2%) were also made. This percentage of mechanically activated C fibers is similar to that reported previously for uninflamed masseter muscle.[26] Given the small number of C fibers, the response properties of all recorded fibers were considered together, and no attempt was made to examine the properties of the C fibers independently. Sixty-five of these fibers (33 in males, 32 in females) were also examined for their response to injection of rat NGF or vehicle control into the masseter muscle.

Baseline Properties of Afferent Fibers

Males. Thirty-three of 71 afferent fiber recordings were made in male rats (mean weight: 301.1 ± 4.9 g; n = 33). The mean baseline MT of these afferent fibers was 23.9 ± 6.0 g, and the mean conduction velocity was 7.3 ± 0.6 m/s. Analysis of these data revealed a significant inverse log-linear correlation between conduction velocity and baseline MT (P = .005, R = 0.473, Fig 2a). The mean plasma estrogen levels of male rats were found to be 22.8 ± 3.1 pg/mL (n = 28). Since slowly conducting Aδ fibers (conduction velocities of ≤ 10 m/s) are more likely

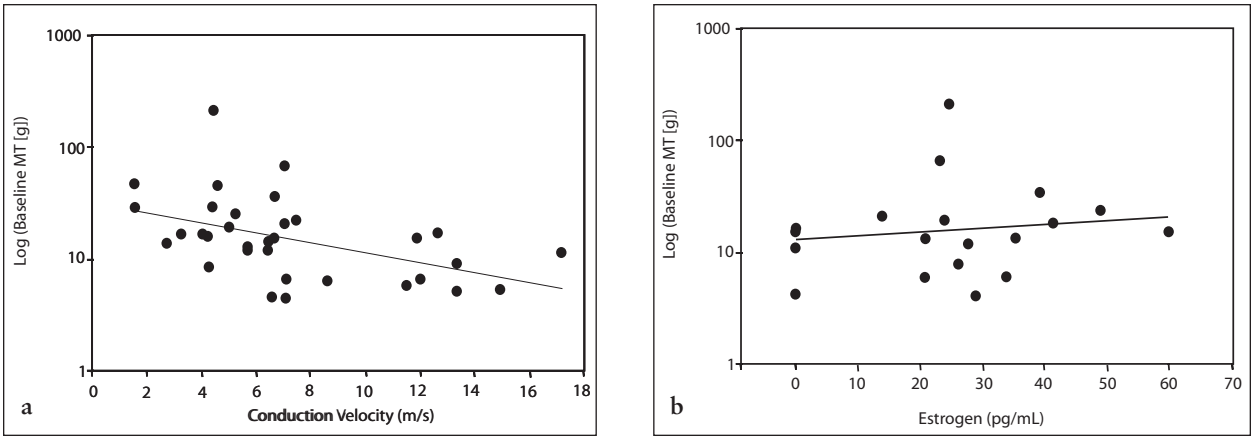

Fig 2 *(a)* The relationship between conduction velocity and baseline MT for fibers recorded in male rats. The log-linear correlation coefficient was calculated to be R = 0.473. *(b)* The relationship between plasma estrogen levels and baseline MT for slow Aδ fibers recorded in male rats is shown. The log-linear correlation coefficient was calculated to be R = 0.187.

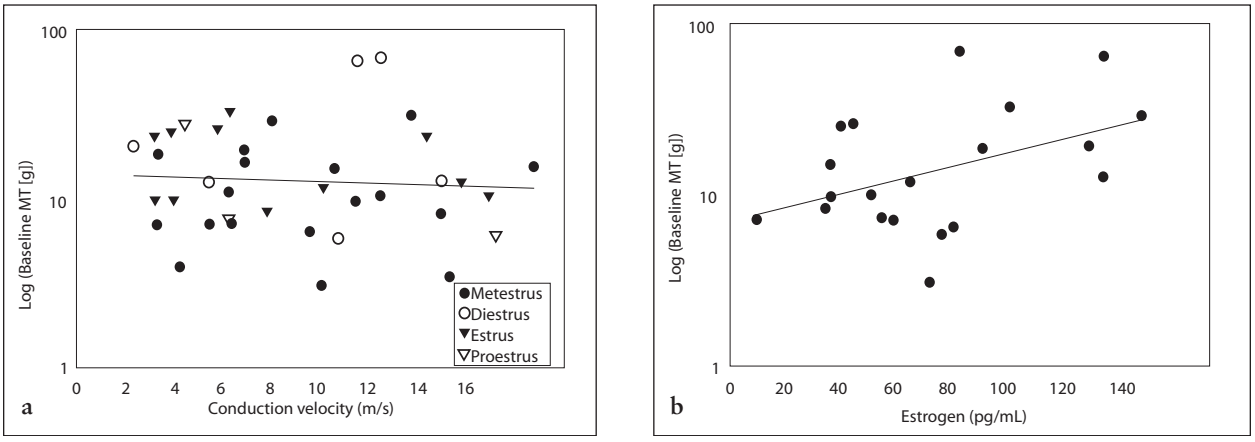

Fig 3 *(a)* The relationship between conduction velocity and baseline MT for fibers recorded in the metestrus, diestrus, estrus, and proestrus stages of female rats. The log-linear correlation coefficient was calculated to be R = 0.07 for all fibers recorded in female rats. *(b)* The relationship between plasma estrogen levels and baseline MT for slow Aδ fibers recorded in female rats is shown. The log-linear correlation coefficient was calculated to be R = 0.444.

to be nociceptive, the relationship between plasma estrogen levels and baseline MT of this subgroup of fibers was examined. There was no significant correlation between the baseline MT and plasma estrogen levels for slow Aδ fibers (P = .43, R = 0.187, Fig 2b). During baseline recording, none of the fibers responded to maximal jaw opening.

Females. Thirty-eight of 71 afferent fiber recordings were made in female rats (weight: 257.5 ± 3.1 g; n = 32). The mean baseline MT of all afferent fibers was 17.0 ± 2.3 g, and the mean conduction velocity was 7.2 ± 0.6 m/s. Unlike in males, the conduction velocity and baseline MT of afferent fibers in females were not log linearly correlated (P = .688, R = 0.07, Fig 3a). Of these 38 afferent fiber recordings, 6 were made during diestrus, 3 during proestrus, 11 during estrus, and 18 during metestrus. There were no significant differences between the mean baseline MTs of fibers recorded during the various estrus stages (P = .124). Baseline MT was not correlated with conduction velocity in any of the individual estrus cycle stages (Fig 3a). The plasma estrogen levels of female rats were found to be 59.0 ± 7.2 pg/mL (n = 26). There was a significant correlation between the baseline MT and plasma estrogen levels for slow Aδ fibers (P = .044, R = 0.444, Fig 3b). During baseline recording, none of the fibers responded to maximum jaw opening.

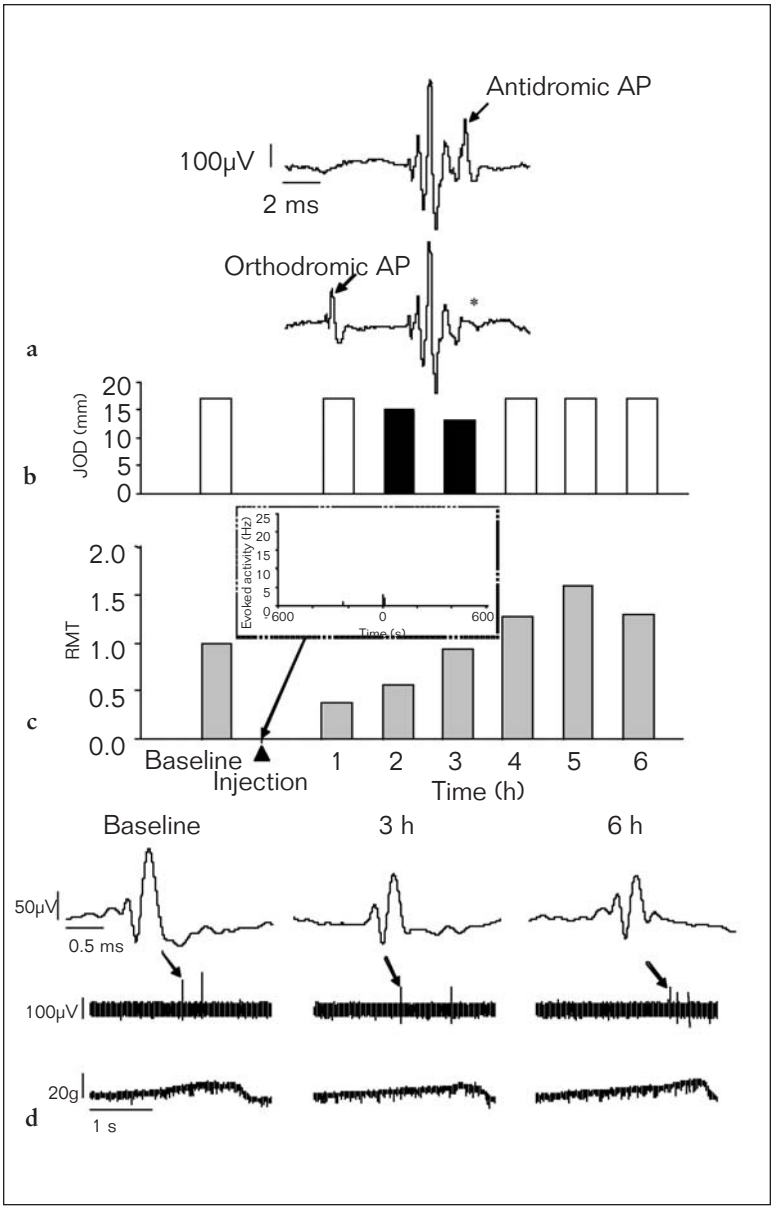

Fig 4 *(a)* An example of a collision between an orthodromic (activated by mechanical stimulation of the masseter muscle) and antidromic (activated by electrical stimulation of the caudal brainstem) action potential (AP) is illustrated. The asterisk (*) indicates where collision resulted in the disappearance of the antidromic spike. This method was used to confirm the projection of the masseter afferent fiber from the masseter muscle to the caudal brainstem. *(b)* JOD over time after a 10-μL injection of 25 μg/mL rat NGF into the masseter muscle. This Aδ fiber (conduction velocity = 4.29 m/s) recorded in a male rat was unresponsive to jaw opening before the injection (white bars) but began firing in response to jaw opening 2 hours after injection (black bars). *(c)* The RMT of the same fiber during baseline and at various time points post-injection. The inset is a histogram of the baseline activity during the 10-minute period before injection and the evoked activity of the fiber in response to the rat NGF injection. Note that there was a transient discharge of APs. This was typical when afferent fibers responded to saline or NGF injections. *(d)* Sample traces of MT recordings from the same fiber at different time points and different time scales during the experiment. The bottom row of traces shows an increase in the force applied with an electronic VF hair to activate masseter muscle fibers (top row of traces). The threshold of the fiber at each time point was determined by subtracting the baseline from the minimum force required to activate the fiber.

Rat NGF-Evoked Responses

Sixty afferent fibers were also examined for their response to injection of vehicle (phosphate-buffered saline) or rat NGF (2.5 or 25 μg/mL) into their mechanoreceptive fields. There was no significant treatment effect on the evoked response of fibers (P = .076). Also, there was no significant change in the mean arterial blood pressure in response to the injection.

In males, relatively few fibers fired 1 or more action potentials in response to injection of vehicle (40%), 2.5 μg/mL rat NGF (40%), or 25 μg/mL rat NGF (10%). Similarly, in females, only 20% of the fibers in each group (vehicle, 2.5 or 25 μg/mL rat NGF) fired 1 or more action potentials in response to the injection. No response outlasted the injection time (~ 5 seconds).

Rat NGF-Induced Changes in Mechanical Properties

Only 1 fiber in the male group (3.3%) responded to jaw opening (25 μg/mL rat NGF injection group) (Fig 4). At 2 hours postinjection, the fiber became responsive to 15 mm of jaw opening, and this effective jaw-opening distance dropped to 13 mm at 3 hours postinjection (~ 24% decrease in effective jaw-opening distance). After 3 hours, the fiber was no longer responsive to maximal jaw opening (Fig 4a). None of the fibers in the female group responded to jaw opening.

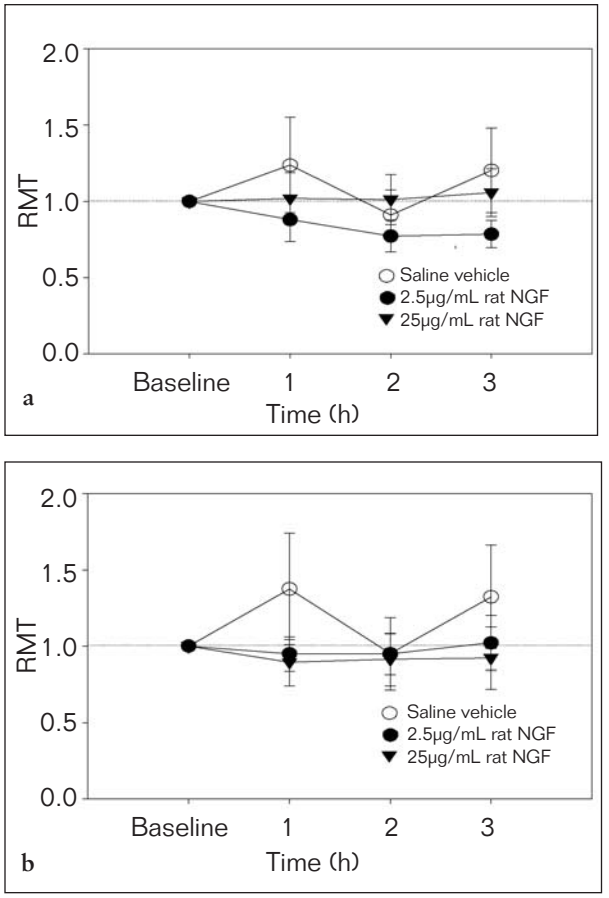

Fig 5 RMTs of fibers from *(a)* male and *(b)* female rats for the first 3 hours after a 10-μL injection of phosphate-buffered saline vehicle (n = 10), 2.5 μg/mL rat NGF (n = 10), and 25 μg/mL rat NGF (n = 10) into the masseter muscle receptive field. The error bars represent the standard error of the mean.

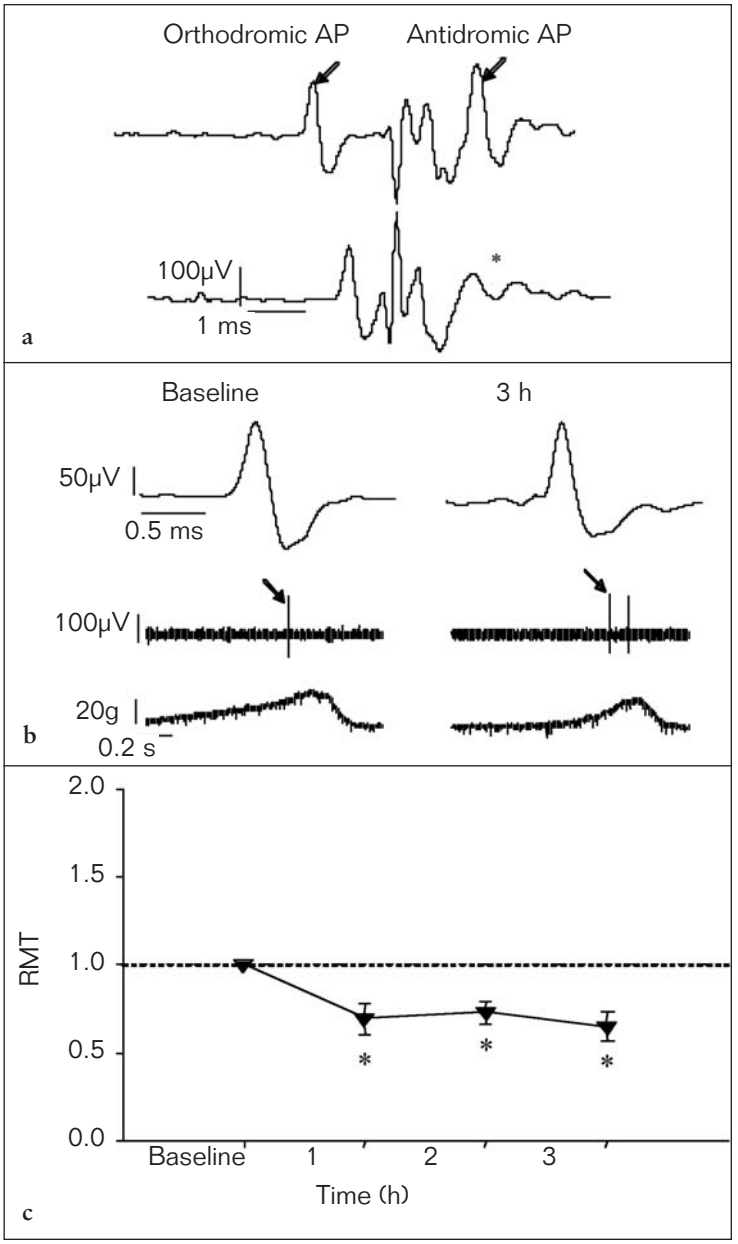

Fig 6 *(a)* An example of a collision between an orthodromic (activated by mechanical stimulation of the masseter muscle) and antidromic (activated by electrical stimulation of the caudal brainstem) action potentials (APs) is illustrated. The asterisk (*) indicates where collision resulted in the disappearance of the antidromic spike. *(b)* Example traces of MT recordings conducted from the same fiber at 2 different time points and 2 different time scales during the experiment. The bottom traces show the force applied with an electronic VF hair to activate masseter muscle fibers (top traces). *(c)* The line graph illustrates the RMTs of fibers from female rats at various time points after a 10-μL injection of 25 μg/mL human NGF (n = 7) into the mechanoreceptive field. The error bars represent the standard error of the mean. $*P \leq .05$ as compared to the baseline RMT.

Although the mechanical threshold of a few afferent fibers was decreased after injection of NGF (Fig 4c), there was no consistent effect of NGF injections on afferent fiber mechanical threshold. Overall, there was no significant treatment, sex or time (repeated) effect on the relative MT, and there were no significant interactions between any of these factors (Fig 5). Also, it was found that there was no correlation between fiber conduction velocity or baseline MT and mean postinjection MT of fibers injected with 2.5-μg/mL or 25-μg/mL NGF.

Evans' Blue Dye Analysis

There was no significant difference in the amount of Evans' blue dye between vehicle and rat NGF treatment groups for either male or female rats, and there was no significant interaction between treatment and sex (P = .643 for treatment, P = .886 for sex, and P = .165 for interaction between treatment and sex). The concentration of Evans' blue dye in vehicle injected muscles was 2.2 ± 0.4 μg/g, which was lower than the values reported previously for normal saline and glutamate injections into the temporomandibular joint (4 hours postinjection).[22] The concentrations of Evans' blue dye in the 2.5-μg/mL and 25-μg/mL groups were 2.1 ± 0.4 μg/g and 1.6 ± 0.3 μg/g, respectively.

Effects of Human NGF

To determine whether there were differences between rat and human NGF that could explain the lack of effect of rat NGF on masseter afferent fibers, additional experiments were conducted with 25 μg/mL human NGF in female rats (mean weight: 250.1 ± 6.1 g; n = 7). The plasma estrogen level of these female rats was 91.1 ± 23.5 pg/mL, and the mean baseline MT and conduction velocity of fibers recorded from these female rats were 23.0 ± 7.4 g and 8.9 ± 1.4 m/s, respectively. The concentration of Evans' blue dye in human NGF injected muscles (2.4 ± 1.3 μg/g) was similar to that found in muscles injected with rat NGF. None of afferent fibers were spontaneously active during the human NGF experiments. One of the 7 masseter fibers fired 1 or more action potentials in response to injection of human NGF into the masseter muscle, and another became responsive to jaw opening at 30 minutes and 1 hour postinjection. In contrast to rat NGF, injection of human NGF into the masseter muscle significantly decreased the relative MT (P = .009, n = 7) at 1, 2, and 3 hours postinjection relative to baseline MT (Fig 6).

Discussion

The principal purpose of this blinded, randomized study was to determine whether NGF significantly alters masseter muscle afferent fiber MT as part of the mechanism whereby it decreases pressure pain thresholds (PPTs) in human subjects. It was also speculated that the discovery that rat NGF injection induced masseter afferent sensitization in rats might lead to the development of a useful animal model to study mechanisms of masseter muscle pain related to myofascial TMD. Injection of either rat or human NGF into the rat masseter muscle did not evoke any significant afferent fiber activity, which is consistent with previous data that injections of human NGF are not painful to humans.[9] Intramuscular injection of rat NGF (2.5 and 25 μg/mL) had no significant effect on the MT of afferent fibers, whereas injection of human NGF decreased the MT of a small population of masseter muscle afferent fibers, which may indicate that human NGF is a more potent sensitizing agent than rat NGF. There was no indication that either human or rat NGF produces these effects indirectly through local muscle inflammation, thus the peripheral effects of NGF are likely mediated through a receptor mechanism, for example TrkA receptor-mediated pathways.[27]

Baseline Properties

Some novel sex-related differences in the baseline characteristics of masseter muscle nociceptors were identified in this study. In agreement with previous studies,[18,26] it was found that there was an inverse relationship between baseline MT and conduction velocity. However, this relationship was only found in the fibers recorded from male rats. Previous studies conducted on male mice and rat cutaneous afferent fibers[28,29] and female guinea pig dorsal root and sural nerve afferents[30] have reported an inverse relationship between baseline MT and conduction velocity, but these studies did not investigate any sex-related differences. Very few studies on muscle afferent fibers have been conducted, and these studies have also shown a similar trend toward an inverse correlation in the relationship between MT and conduction velocity.[18,31] The results of this study agree with these previous reports and also indicate that there are sex-related differences in the relationship between baseline MT and conduction velocity.

Another unexpected finding was that the plasma estrogen levels of normally cycling female rats were positively correlated with the baseline MT of slow

Aδ fibers. In contrast, previous studies in gonadectomized rats have shown that 9 to 10 days of exogenous estrogen administration increases the excitability of facial cutaneous afferent fibers and enhances nociceptive jaw-reflex responses,[32,33] which may indicate that elevated blood levels of estrogen are pronociceptive. The findings of the present study suggest that under normal physiological conditions, elevation of estrogen levels is associated with decreased excitability of putative masticatory muscle nociceptors. Such an association may provide a mechanistic basis for the observation of increased TMD pain at menstruation (when estrogen levels are low) in some women with TMD.[6]

Evoked Responses

The responses evoked by rat or human NGF were not significantly different from those evoked by vehicle injection. Human subjects did not find injection of human NGF into the masseter muscle painful.[9] Indeed, previous work has established a relationship between chemically evoked masseter afferent discharges of slower-conducting fibers (slow Aδ and C fibers) in rats and muscle pain in humans upon injection of the same chemical algogen.[18,26,34] Therefore, the lack of responses of slow Aδ and C fibers in this study to rat NGF injection is consistent with the interpretation that intramuscular injection of rat NGF does not evoke muscle pain.

Mechanical Properties

This study was undertaken to investigate whether intramuscular rat NGF injection significantly alters masseter muscle afferent fiber MT as part of the mechanism whereby it decreases PPTs in human subjects. It has been reported that intravenous and subcutaneous/intradermal administration of human NGF results in dose-dependent muscle pain symptoms in the craniofacial and trunk musculature as well as the masseter muscle that increased with function.[35,36] Further, men and women have reported localized mechanical sensitization after injection of human NGF into the masseter muscle that lasts from 7 to 14 days; in women, the onset of mechanical sensitization occurs within 3 hours of NGF injection.[9] Thus, the lack of effect of intramuscular injection of rat NGF on the MT of rat masseter afferent fibers was unexpected. The additional experiments conducted with human NGF showed that human NGF caused mechanical sensitization that lasted for at least 3 hours, which suggests that human NGF is more effective at sensitizing rat masseter muscle fibers than rat NGF. Rat and human NGF share only 90% amino acid sequence homology,[37,38] so it is possible that structural differences might underlie the apparent differences in efficacy and/or potency of these 2 neurotrophic proteins. It is possible that nonspecific mechanisms such as foreign protein reactions, decreased degradation of human NGF by rat proteases, slowed clearance from the muscle, modulation of intracellular calcium concentration (calcium uptake),[39,40] or actions on bradykinin receptors,[41] capsaicin receptors,[25,42] or sodium channels[43,44] might contribute to the observed difference. Particularly intriguing is a recent study which found that NGF can enhance NMDA-evoked currents in cultured murine and isolated rat hippocampal neurons lacking TrkA receptors,[45] since activation of peripheral NMDA receptors can mechanically sensitize the masseter muscle in humans and masseter afferent fibers in rats.[18,46]

It may also be that species, differences in the expression or density of TrkA and/or p75 receptors on masseter muscle afferent fibers contribute to the observed effects of NGF administration. About 65% of the cell bodies of primary sensory neurons of human peripheral ganglia and skin express the TrkA receptor.[47] In adult rats, only 40% and 50% of dorsal root ganglion neurons (small- and medium-sized cell bodies) express the high-affinity TrkA and low-affinity p75 receptors, respectively,[48–52] and the expression of TrkA receptors in putative nociceptive trigeminal ganglion neurons has been reported to be even lower.[49] Only 10% of neurons innervating the incisor and 15% of corneal neurons were found to be TrkA positive in adult and neonatal mice.[49] These studies suggest that tissue and species-related differences in the expression of NGF receptors exist. Perhaps only a small percentage of masseter afferent fibers express the TrkA receptor, and thus only a few fibers would be sensitized by NGF.

Conclusions

The results of this blinded, randomized study suggest that intramuscular injection of rat NGF at a concentration equivalent to that which resulted in mechanical sensitization in humans did not cause any significant change in the MT of putative nociceptive rat masseter muscle afferent fibers. The reasons for the lack of effect of rat NGF on masseter muscle afferent fibers are as yet unclear, but it was demonstrated that human NGF is significantly more effective than rat NGF as an inducer of masseter muscle afferent sensitization in rats. In

this regard, injection of human NGF into the rat masseter muscle offers a better method for the study of peripheral mechanisms associated with the development of NGF-induced masseter muscle sensitization in humans.

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References

1. LeResche L. Epidemiology of orofacial pain. In: Lund JP, Lavigne GJ, Dubner R, Sessle BJ (eds). Orofacial Pain: From Basic Science to Clinical Management. Chicago: Quintessence, 2001:15–26.
2. McNeill C, Dubner R. What is pain and how do we classify orofacial pain? In: Lund JP, Lavigne GJ, Dubner R, Sessle BJ (eds). Orofacial Pain: From Basic Science to Clinical Management. Chicago: Quintessence, 2001:3–14.
3. Dworkin SF, LeResche L. Research Diagnostic Criteria for Temporomandibular Disorders: Review, criteria, examinations and specifications, critique. J Craniomandib Disord 1992;6:301–355.
4. Nekora-Azak A. Temporomandibular disorders in relation to female reproductive hormones: A literature review. J Prosthet Dent 2004;91:491–493.
5. LeResche L, Saunders K, Von Korff MR, Barlow W, Dworkin SF. Use of exogenous hormones and risk of temporomandibular disorder pain. Pain 1997;69:153–160.
6. LeResche L, Mancl L, Sherman JJ, Gandara B, Dworkin SF. Changes in temporomandibular pain and other symptoms across the menstrual cycle. Pain 2003;106:253–261.
7. Isselee H, De Laat A, De Mot B, Lysens R. Pressure-pain threshold variation in temporomandibular disorder myalgia over the course of the menstrual cycle. J Orofac Pain 2002;16:105–117.
8. Stohler CS. Masticatory myalgias. Emphasis on the nerve growth factor-estrogen link. Pain Forum 1997;6:176–180.
9. Svensson P, Cairns BE, Wang K, Arendt-Nielsen L. Injection of nerve growth factor into human masseter muscle evokes long lasting mechanical allodynia and hyperalgesia. Pain 2003;104:241–247.
10. Chao MV. Neurotrophins and their receptors: A convergence point for many signaling pathways. Nat Rev Neurosci 2003;4:299–309.
11. Dmitrieva N, McMahon SB. Sensitisation of visceral afferents by nerve growth factor in the adult rat. Pain 1996;66:87–97.
12. Djouhri L, Dawbarn D, Robertson A, Newton R, Lawson SN. Time course and nerve growth factor dependence of inflammation-induced alterations in electrophysiological membrane properties in nociceptive primary afferent neurons. J Neurosci 2001;21:8722–8733.
13. Rueff A, Dawson AJ, Mendell LM. Characteristics of nerve growth factor induced hyperalgesia in adult rats: Dependence on enhanced bradykinin-1 receptor activity but not neurokinin-1 receptor activation. Pain 1996; 66:359–372.
14. Rueff A, Mendell LM. Nerve growth factor and NT-5 induce increased thermal sensitivity of cutaneous nociceptors in vitro. J Neurophysiol 1996;76:3593–3596.
15. Thompson SWN, Dray A, McCarson KE, Krause JE, Urban L. Nerve growth factor induces mechanical allodynia associated with novel A fibre-evoked spinal reflex activity and enhanced neurokinin-1 receptor activation in the rat. Pain 1995;62:219–231.
16. Lewin GR, Ritter AM, Mendell LM. Nerve growth factor-induced hyperalgesia in the neonatal and adult rat. J Neurosci 1993;13:2136–2148.
17. Lewin GR, Rueff A, Mendell LM. Peripheral and central mechanisms of NGF-induced hyperalgesia. Eur J Neurosci 1994;6:1903–1912.
18. Cairns BE, Gambarota G, Svensson P, Arendt-Nielsen L, Berde CB. Glutamate-induced sensitization of rat masseter muscle fibers. Neuroscience 2002;109:389–399.
19. Arvidsson J, Raappana P. An HRP study of the central projections from primary sensory neurons innervating the rat masseter muscle. Brain Res 1989;480:111–118.
20. Capra NF, Wax TD. Distribution and central projections of primary afferent neurons that innervated the masseter muscle and mandibular periodontium: A double-label study. J Comp Neurol 1989; 279:341–352.
21. Shigenaga Y, Sera M, Nishimori T, et al. The central projection of masticatory afferent fibers to the trigeminal sensory nuclear complex and upper cervical spinal cord. J Comp Neurol 1988;268:489–507.
22. Fiorentino PM, Cairns BE, Hu JW. Development of inflammation after application of mustard oil or glutamate to the rat temporomandibular joint. Arch Oral Biol 1999;44:27–32.
23. Svensson P, Cairns BE, Wang K, et al. Glutamate-evoked pain and mechanical allodynia in the human masseter muscle. Pain 2003;101:221–227.
24. Andreev NY, Dimitrieva N, Koltzenburg M, McMahon SB. Peripheral administration of nerve growth factor in the adult rat produces a thermal hyperalgesia that requires the presence of sympathetic post-ganglionic neurons. Pain 1995;63:109–115.
25. Chuang H, Prescott ED, Kong H, et al. Bradykinin and nerve growth factor release the capsaicin receptor from PtdIns(4,5)P2-mediated inhibition. Nature Rev 2001; 411:957–962.
26. Cairns BE, Svensson P, Wang K, et al. Activation of peripheral NMDA receptors contributes to human pain and rat afferent discharges evoked by injection of glutamate into the masseter muscle. J Neurophysiol 2003; 90:2098–2105.
27. Malik-Hall M, Dina OA, Levine JD. Primary afferent nociceptor mechanisms mediating NGF-induced mechanical hyperalgesia. Eur J Neurosci 2005;21:3387–3394.
28. Cain DM, Khasabov SG, Simone DA. Response properties of mechanoreceptors and nociceptors in mouse glabrous skin: An in vivo study. J Neurophysiol 2001;85:1561–1574.
29. Du J, Koltzenburg M, Carlton SM. Glutamate-induced excitation and sensitization of nociceptors in rat glabrous skin. Pain 2001;89:187–198.
30. Djouhri L, Lawson SN. Increased conduction velocity of nociceptive primary afferent neurons during unilateral hindlimb inflammation in the anaesthetised guinea-pig. Neuroscience 2001;102:669–679.
31. Ge W, Khalsa PS. Encoding of compressive stress during indentation by group III and IV muscle mechano-nociceptors in rat gracilis muscle. J Neurophysiol 2003;89:785–792.

32. Bereiter DA, Barker DJ. Facial receptive fields of trigeminal neurons: Increased size following estrogen treatment in female rats. Neuroendocrinology 1975;18:115–124.
33. Cairns BE, Sim Y-L, Bereiter DA, Sessle BJ, Hu JW. Influence of sex on reflex jaw muscle activity evoked from the rat temporomandibular joint. Brain Res 2002;957: 338–344.
34. Cairns BE, Hu JW, Arendt-Nielsen L, Sessle BJ, Svensson P. Sex-related differences in human pain perception and rat afferent discharge evoked by injection of glutamate into the masseter muscle. J Neurophysiol 2001;86: 782–791.
35. Dyck PJ, Peroutka S, Rask C, et al. Intradermal recombinant human nerve growth factor induces pressure allodynia and lowered heat-pain threshold in humans. Neurology 1997;48:501–505.
36. Petty BG, Cornblath DR, Adornato BT, et al. The effect of systemically administered recombinant human nerve growth factor in healthy human subjects. Ann Neurol 1994;36:244–246.
37. Whittemore SR, Friedman PL, Larhammar D, Persson H, Gonzalez-Carvajal M, Holets VR. Rat beta-nerve growth factor sequence and site of synthesis in the adult hippocampus. J Neurosci Res 1988;20:403–410.
38. Wiesmann C, de Vos AM. Nerve growth factor: Structure and function. Cell Mol Life Sci 2001;58:748–759.
39. Jiang H, Takeda K, Lazarovici P, et al. Nerve growth factor (NGF)-induced calcium influx and intracellular calcium mobilization in 3T3 cells expressing NGF receptors. J Biol Chem 1999;274:26209–26216.
40. Jiang H, Guroff G. Actions of the neurotrophins on calcium uptake. J Neurosci Res 1997;50:355–360.
41. Dray A, Perkins M. Bradykinin and inflammatory pain. Trends Neurosci 1993;16:99–104.
42. Zhu W, Galoyan SM, Petruska JC, Oxford GS, Mendell LM. A developmental switch in acute sensitization of small dorsal root ganglion (DRG) neurons to capsaicin or noxious heating by NGF. J Neurophysiol 2004;92: 3148–3152.
43. Fjell J, Cummins TR, Fried K, Black JA, Waxman SG. In vivo NGF deprivation reduces SNS expression and TTX-R sodium currents in IB4-negative DRG neurons. J Neurophysiol 1999;81:803–810.
44. Fjell J, Cummins TR, Dib-Hajj SD, Fried K, Black JA, Waxman SG. Differential role of GDNF and NGF in the maintenance of two TTX-resistant sodium channels in adult DRG neurons. Brain Res Mol Brain Res 1999;67: 267–282.
45. Jarvis CR, Xiong ZG, Plant JR, et al. Neurotrophin modulation of NMDA receptors in cultured murine and isolated rat neurons. J Neurophysiol 1997;78:2363–2371.
46. Cairns BE, Svensson P, Wang K, et al. Ketamine attenuates glutamate-induced mechanical sensitization of the masseter muscle in human males. Exp Brain Res 2006; 169:467–472.
47. Vega JA, Vazquez E, Naves FJ, Del Valle ME, Calzada B, Represa JJ. Immunohistochemical localization of the high-affinity NGF receptor (gp140-trkA) in the adult human dorsal root and sympathetic ganglia and in the nerves and sensory corpuscles supplying digital skin. Anat Rec 1994;240:579–588.
48. Bennett DL, Averill S, Clary DO, Priestley JV, McMahon SB. Postnatal changes in the expression of the trkA high-affinity NGF receptor in primary sensory neurons. Eur J Neurosci 1996;8:2204–2208.
49. Mosconi T, Snider WD, Jacquin MF. Neurotrophin receptor expression in retrogradely labeled trigeminal nociceptors–-comparisons with spinal nociceptors. Somatosens Mot Res 2001;18:312–321.
50. Averill S, McMahon SB, Clary DO, Reichardt LF, Priestley JV. Immunocytochemical localization of trkA receptors in chemically identified subgroups of adult rat sensory neurons. Eur J Neurosci 1995;7:1484–1494.
51. Molliver DC, Radeke MJ, Feinstein SC, Snider WD. Presence or absence of TrkA protein distinguishes subsets of small sensory neurons with unique cytochemical characteristics and dorsal horn projections. J Comp Neurol 1995;361:404–416.
52. Fang X, Djouhri L, McMullan S, et al. trkA is expressed in nociceptive neurons and influences electrophysiological properties via Nav1.8 expression in rapidly conducting nociceptors. J Neurosci 2005;25:4868–4878.