Nerve Growth Factor Increases Glutamate in Sensory Fibres Innervating the Masseter Muscles of Female Rats

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Objective: *To investigate whether nerve growth factor (NGF) alters glutamate expression in sensory fibres and glutamate concentration in the masseter muscle of female rats.*
Methods: *Ten female rats were injected with NGF (25 μg/ml, 10 μl) and vehicle into the right and left masseter muscles, respectively. Immunohistochemistry and microdialysis were performed after 3 days to evaluate glutamate expression and concentration in the muscle.*
Results: *The frequency of expression of glutamate in the nerve fibres innervating the masseter muscle was significantly greater 3 days after NGF (56 ± 5%) than after vehicle (39 ± 5%) injection. The majority of fibres co-expressed the neuropeptide substance P (SP); a marker for sensory afferent fibres. There was no effect of NGF on the expression of the excitatory amino acid transporter type 2 (EAAT2). In the microdialysis experiment, mean interstitial glutamate concentration on the vehicle side (21.6 ± 9.8 μM) was not significantly different from that on the NGF side (16.2 ± 9.2 μM).*
Conclusion: *These results suggest that, in part, NGF increases the mechanical sensitivity of the masseter muscle by increasing glutamate expression in the sensory nerve endings in the muscle. This effect was local to the site of the NGF injection, as it was only detectable through immunohistochemistry, but not by microdialysis.*
Key words: *glutamate, nerve growth factor, sensory fibre, temporomandibular disorders*
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Temporomandibular disorders (TMD) are a group of musculoskeletal pain conditions of the head and neck that affect a significant segment of the population[1,2]. A number of human and animal studies suggested peripheral glutamate might play a role in masticatory muscle pain in TMD patients[3-5]. In particular, interstitial glutamate concentrations were higher in the masseter muscles of myofascial TMD patients when compared with healthy controls[3]. Injection of high concentration glutamate into the masseter muscle of healthy subjects induced pain and mechanical sensitisation and this response was blocked by a N-methyl-D-aspartate (NMDA) receptor antagonist[4,5]. In human experiments, ingestion of monosodium glutamate increased muscle sensitivity and interstitial glutamate concentrations in the masseter muscles of healthy subjects and TMD patients[6,7]. In rat masseter muscle, baseline interstitial glutamate concentration was found to be ~20 μM. Increasing interstitial glutamate concentration in the masseter muscle two to three times via systemic injection of monosodium glutamate resulted in a decrease in the mechanical threshold of masseter muscle nociceptors and this effect was inhibited by NMDA receptor antagonists[8]. These results suggest that the peripheral glutamate system may play an important role in regulating mechanical sensitivity of the masseter muscle.

NGF is a neurotrophin essential for the development, maintenance and regeneration of sympathetic and small-diameter sensory afferent fibres. It is also an important mediator of peripheral nociception[9]. In humans and rats, intramuscular injection of NGF has been used to model myofascial TMD[10]. It decreases the mechanical threshold of masseter muscle mechanonociceptors, in part, by increasing the expression of NMDA glutamate receptors in peripheral endings[11]. Since it has been found that primary afferent fibres may also release glutamate upon stimulation, lowering the activation threshold may result in a positive feedback cycle of glutamate-NMDA receptor sensitisation in

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masseter muscle nociceptors[12-14]. NGF has also been shown, *in vitro*, to induce glutamate release from cultured neurons and trigeminal satellite glial cells[15-17].

Two to three times more women than men suffer from TMD[2]. Also, glutamate-evoked pain was greater in women than men[18]. This sex-related difference was mimicked by NGF-induced muscle sensitisation in the rats with greater sensitivity in the female rats than in the male rat, accompanied by greater upregulation of peripheral NMDA receptors in the trigeminal neurons innervating the masseter muscles[11]. Increased expression of neuropeptides (calcitonin gene-related peptide, CGRP and SP) was also only observed in the female rats. Here we used female rats to better represent the patient population, as well as to further study the potential role of the peripheral glutamatergic system in these sex-related differences. In this study, we used immunohistochemistry and microdialysis to investigate whether NGF alters the glutamate concentration in the masseter muscle of female rats.

Materials and methods

Animals

Ten female (245 g to 314 g) Sprague-Dawley rats were used. Animals were housed in pairs with a 12 h light/dark cycle. Food and water were given *ad libitum*. All animal procedures were reviewed and approved by the University of British Columbia Animal Care Committee.

Immunohistochemistry

Five female rats were injected with NGF (25 µg/ml, 10 µl, Sigma, St Louis, MO, USA) or vehicle (phosphate buffered saline, PBS, 10 µl) into the left and right masseter muscles, respectively.

Three days after injection, the animals were perfused with 120 ml cold saline followed by 120 ml of paraformaldehyde (4%) under deep isoflurane anaesthesia. The concentration of NGF and the method of injection were based on a previous rat behavioural experiment where it was found that NGF induced mechanical sensitisation through upregulation of peripheral NMDA receptor and neuropeptides, which peaked 3 days after injection[11]. After intramuscular injections, a permanent marker for subsequent identification marked the sites of injection. Masseter muscle sections of 10 mm × 10 mm × 10 mm around the sites of injection were removed. Expression levels of glutamate, EAAT2, SP were compared between the NGF-injected muscles and the PBS-injected muscles on the contralateral side. Masseter muscles were incubated in 20% and 40% sucrose for 48 h each to cryoprotect the tissue, and cut into 10 µm sections with a cryotome. Sections were treated with 5% normal goat serum (NGS) in PBS for 1 h and incubated overnight with commercial available primary antibodies against the neuronal marker protein gene product 9.5 (PGP9.5; 1/1000; rabbit; Cat#: PA1-46343; Thermo Scientific, Rockford IL, USA), the excitatory amino acid transporter EAAT2 (1/250; goat; Cat#: sc-7760; Santa Cruz Biotechnology, Dallas Texas, USA), and substance P (1/700; guinea pig; Cat#: ab10353; ABCAM, Cambridge MA, USA) or glutamate (1/100; chicken; Cat# ab62668; ABCAM, Cambridge MA, USA). The next morning sections were washed several times with PBS and then incubated for 1 h at room temperature in the dark in the presence of appropriate secondary antibodies with fluorescent tracers attached (donkey anti-goat Alexa Fluor 488, donkey anti-rabbit Alexa Fluor 555 and goat anti-chicken Alexa Fluor 633 or goat anti-guinea pig Alexa Fluor 633; 1/700; Goat antibodies were applied separately after the donkey antibodies had been incubated and washed to avoid cross-reactivity). After several washes in buffer, all sections were mounted on slides with covers slips and visualised with a Leica TCS SPE confocal microscope. Images were analysed using the image-processing programme ImageJ (National Institute of Health, USA). As the study was not blinded, the following quantification method was vigorously followed to limit potential bias. An area from the image with non-specific binding was selected as the background. The mean and standard deviation for this area were calculated from the brightness values of the pixels by the ImageJ programme. Nerve fibres were considered positive when the intensity of the fluorescent tracer signal for PGP 9.5 exceeded the two standard deviations of the mean background intensity. The minimum accepted length for a PGP 9.5-labelled nerve fibre was 1 µm in the masseter muscle. Omission of the primary antibodies was performed as a control for nonspecific binding of the secondary antibodies.

The anti-glutamate antibody was raised in chicken against L-glutamate conjugated to glutaraldehyde as the immunogen peptide. It was characterised against a spectrum of antigens to assure hapten selectivity by the manufacturer (manufacturer's datasheet). The manufacturer's information shows no measurable cross-reactivity against glutamate in peptides or proteins and other amino acids (1000×) except modest cross-reactivity against

D-glutamate (20×). This method of using anti-glutamate antibodies raised against L-glutamate conjugated to glutaraldehyde for fluorescence detection of free glutamate in paraformaldehyde-fixed neural tissues has been published and validated previously in the literature[19,20]. The specificity of the anti-EAAT2 antibody has been validated previously using knockout mice, while the specificity of the anti-SP antibody has been confirmed by preabsorption with synthetic peptid[21,22].

Microdialysis

Five rats received injections of NGF (25 μg/ml, 10 μl) and vehicle (PBS) into the right and left masseter muscles, respectively, under brief isoflurane anaesthesia. Three days after injection, the rats were then anesthetised with isoflurane (2% to 2.5% in oxygen 97% to 98%; AErrane; Baxter). Previously, it was found that NGF-induced NMDA receptor upregulation peaked 3 days after intramuscular injection[11]. Heart rate, blood pressure and body temperature were monitored and a trachea tube was inserted for ventilation throughout the experiment. Microdialysis was performed as previously described[8]. Microdialysis probes (MAB 1.2.4. PES with 6kDa cut-off, Microbiotech, Stockholm, Sweden) were inserted into the muscles at the site of injection. A small incision (~5 mm) was made to the skin over the site of injection at the masseter muscle to allow for insertion of the microdialysis probe. The probes were perfused with pH 7.4 phosphate buffered saline (PBS) by a microinfusion pump (MAB 20 microdialysis pump, WM Altea AB, Sweden) at a rate of 2 μl/min. An initial stabilisation period of 60 min was followed by a sampling period of 120 min where two 100 μl samples of dialysate were collected. A pilot study was performed prior to the experiments showing that glutamate concentrations in the dialysates stabilised following a 60 min period (data not shown). After the sampling period ended, the microdialysis probe was perfused with a solution of 100 μM monosodium glutamate to determine the glutamate recovery rate in each probe. Glutamate recovery was estimated by dividing the concentration of glutamate collected from the probe divided by 100 μM, the initial concentration of glutamate in the dialysate. The average glutamate recovery rate of the probes was 0.74 ± 0.06. Glutamate concentration in the dialysate was determined by an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (LDN Labor Diagnostika Nord GmbH & Co. KG, Nordhorn, Germany; Cat No: BA E-2300). Briefly, the competitive ELISA used the microtiter plate format. The antigen was bound to the solid phase of the microtiter plate. The acylated analyte concentrations in the samples and the solid phase bound analyte competed for a fixed number of antibody binding sites. After equilibrium was reached, the antibody bound to the solid phase was detected by an anti-rabbit IgG-peroxidase conjugate using tetramethylbenzidine as a substrate. The reaction was monitored at 450 nm. Quantification of the samples was achieved by comparing their absorbance with a standard curve prepared with standards provided by the manufacturer. Concentrations were then corrected by the relative recovery rate of the dialysis probe to provide an estimate of muscle interstitial glutamate concentration.

Data analysis

For the immunohistochemistry experiment, the frequency of expression of EAAT2, SP and glutamate in PGP9.5-positive fibres in the masseter muscle between NGF and PBS-injected sides was analysed using a pairwise Student *t* test. Glutamate concentrations collected from microdialysis from the PBS-injected and NGF-injected sides were analysed with the paired Student *t* test. A paired *t* test sample size estimation indicated that to detect a minimum difference of 100%, with an estimated standard deviation of the difference of 50%, and $\alpha = 0.05$, and $\beta = 0.20$, a minimum of four animals per group were needed. A previous study found that a two- to threefold increase in interstitial glutamate concentration was required to increase masseter muscle sensitivity[8]. A probability level of 0.05 was considered significant for all tests. Error bars represented standard error of the mean.

Results

Immunohistochemistry

Immunohistochemistry was performed to investigate whether nerve fibres innervating the masseter muscle express the excitatory amino acid transporter EAAT2 and co-localisation with SP was used to determine whether they were sensory fibres (Figs 1 and 2). The frequency of expression of EAAT2 was 56 ± 9% and expression of SP was 29 ± 7% in the PBS-injected muscle. NGF did not significantly alter the expression of EAAT2, but increased the expression of SP, with a frequency of expression of 72 ± 4% and 53 ± 7% for EAAT2 and SP, respectively. Co-localisation of EAAT2 with SP was found to be ~80% and was unaffected by NGF, suggesting that the majority of sensory fibres expressing SP also express EAAT2 (Fig 2B).

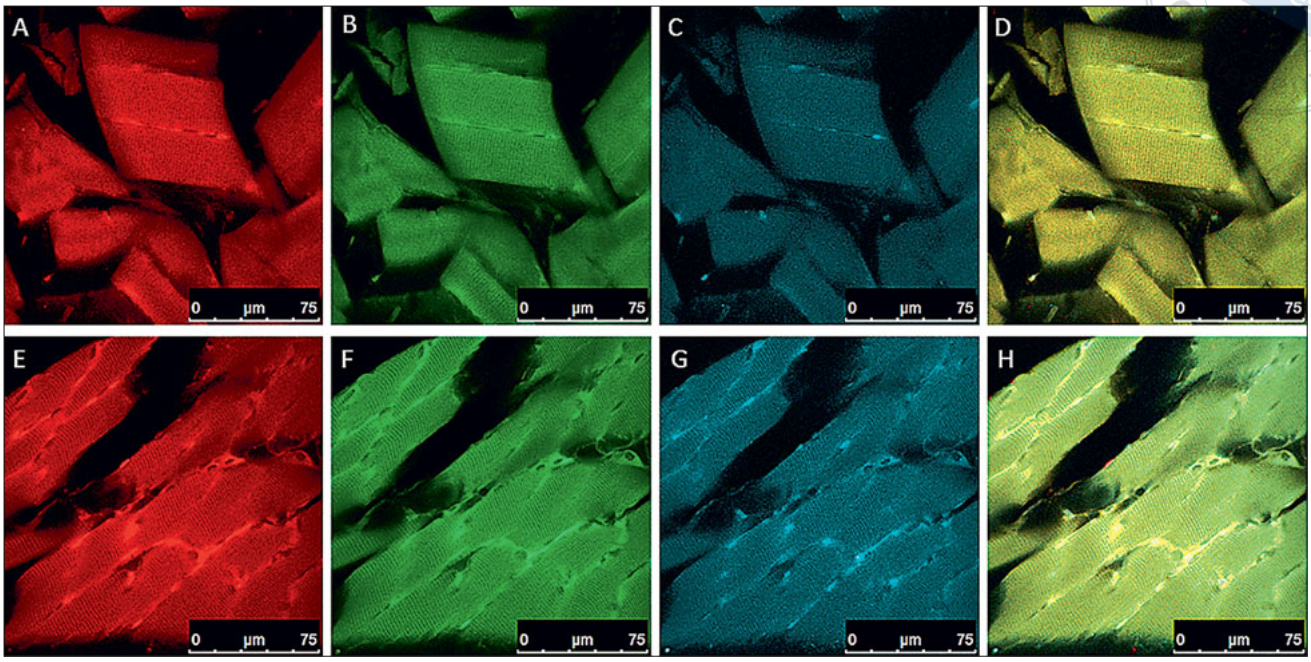

Fig 1 Example photomicrographs of two sections of rat masseter muscle (n = 5) 3 days after PBS **(A-D)** or NGF injection **(E-H)**. Nerve fibres were identified by PGP9.5 antibody **(A, E)**, and tested for expression of EAAT2 **(B, F)**, and Substance P **(C, G)**. **D** and **H** are composite of all three labels. Scale bar = 75 µm.

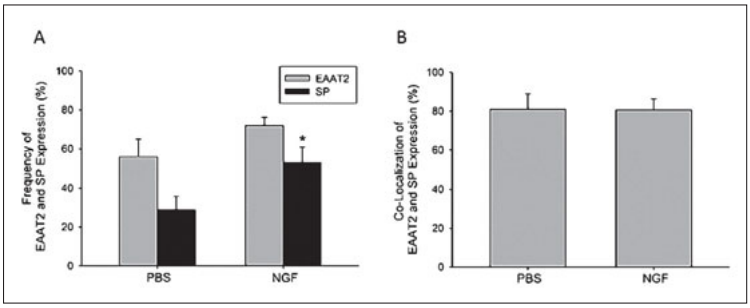

Fig 2 **(A)** The bar graph shows the frequency of expression of EAAT2 and SP in nerve fibres innervating the masseter muscle following intramuscular injections of PBS or NGF (n = 5); **(B)** The bar graph shows the co-localisation of EAAT2 and SP in these fibres. The asterisk denotes a significant difference between the NGF-injected side and the vehicle-injected side by paired Student *t* tests ($P < 0.05$).

Expression of glutamate and EAAT2 in PBS and NGF-injected muscles is shown in Figures 3 and 4. The frequency of expression of glutamate was 39 ± 5% in the PBS-injected side and was significantly higher in the NGF-injected side, with a frequency of expression of 56 ± 5%. Co-localisation of glutamate and EAAT2 was found to be ~80% and was unaffected by NGF (Fig 4B).

Microdialysis

Microdialysis experiments were undertaken to determine whether the increased expression of glutamate in the muscle nerve fibres after NGF treatment affected interstitial glutamate concentrations. Glutamate was collected from the female masseter muscles, as shown in Figure 5A, 3 days after intramuscular injections of PBS or NGF. The estimated interstitial glutamate concentrations were 21.6 ± 9.8 µM in the PBS-injected side and 16.2 ± 9.2 µM in the NGF-injected side (Fig 5B). The difference between the PBS and NGF-injected sides was not significant ($P > 0.05$).

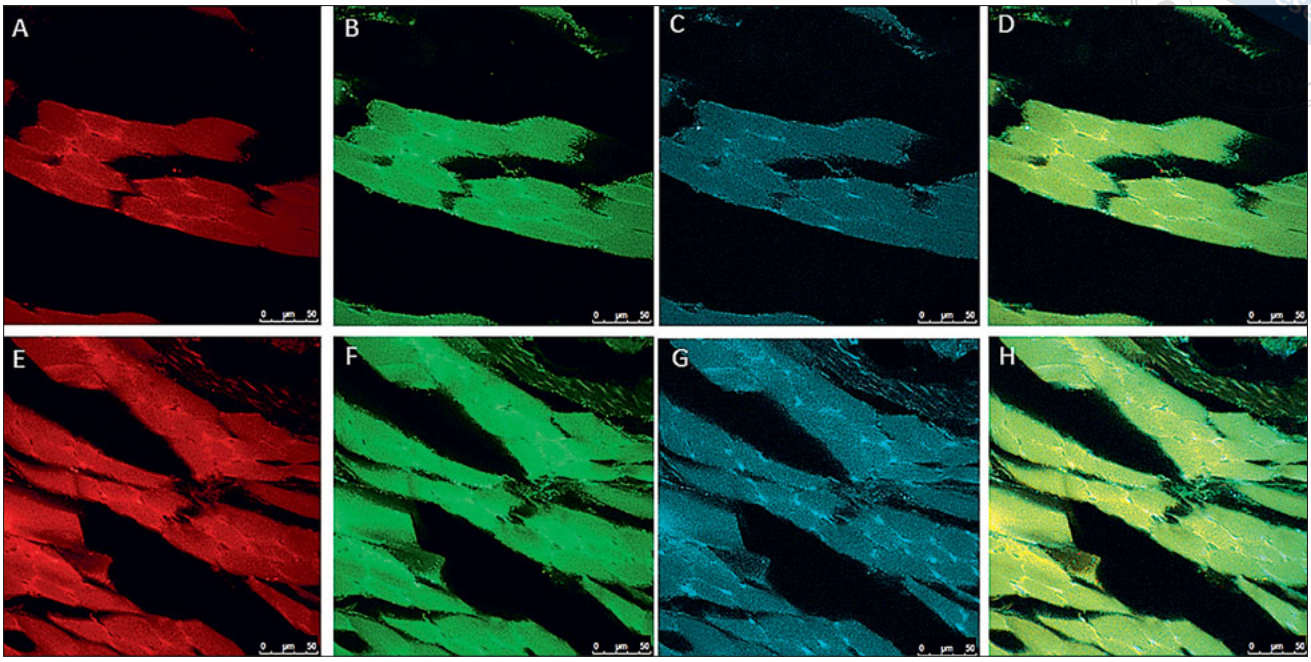

Fig 3 Example photomicrographs of two sections of rat masseter muscle (n = 5) 3 days after PBS **(A-D)** or NGF injection (E-H). Nerve fibres were identified by PGP9.5 antibody **(A, E)**, and tested for expression of EAAT2 **(B, F)**, and glutamate **(C, G)**. **D** and **H** are composite of all three labels. Scale bar = 75 μm.

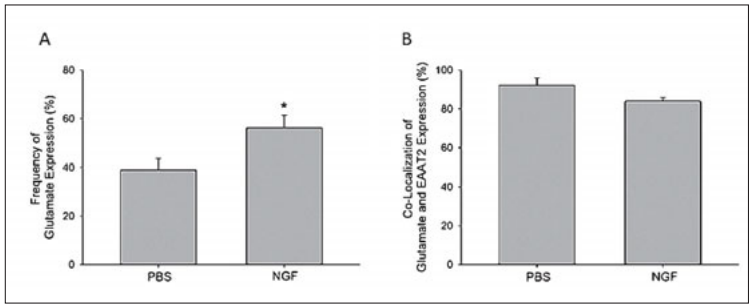

Fig 4 **(A)** The bar graph shows the frequency of expression of EAAT2 and glutamate in nerve fibres innervating the masseter muscle following intramuscular injections of PBS or NGF (n = 5). **(B)** The bar graph shows the co-localisation of EAAT2 and glutamate in these fibres. The asterisk denotes a significant difference between the NGF-injected side and the vehicle-injected side by paired Student *t* tests ($P < 0.05$).

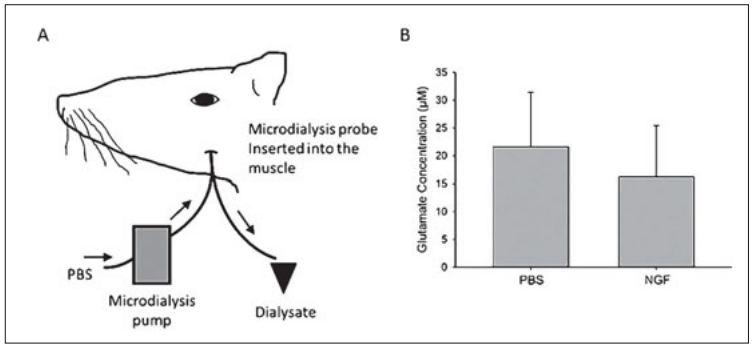

Fig 5 **(A)** The experimental setup to determine the interstitial concentration of glutamate in the rat masseter muscle by microdialysis. **(B)** The bar graph shows the interstitial glutamate concentration in the masseter muscles of female rats (n = 5) at 3 days after PBS and NGF injection. No significant difference was observed between the NGF-injected side and the vehicle-injected side by paired Student *t* tests ($P > 0.05$).

Discussion

A higher interstitial concentration of glutamate was previously found in the masseter muscles of TMD patients[3]. *In vitro* studies showed that NGF could induce glutamate release from cultured neurons, suggesting a potential interaction between NGF and peripheral glutamate release[15,16]. Here we found intramuscular injection of NGF increased glutamate expression in peripheral nerve fibres innervating the masseter muscles of female rats. The majority of these fibres expressed EAAT2 and SP, suggesting that these were peripheral sensory fibres. In an earlier study, we found that NGF-induced sensitisation in the masseter muscles is mediated by an increased expression of NMDA receptors in the peripheral endings of mechano-nociceptors innervating the masseter muscles of male and female rats[11,23]. Taken together, our results suggest that NGF induces mechanical sensitisation in the masseter muscle, in part by acting on the peripheral glutamatergic system through inducing localised glutamate release and increasing receptor expression.

In the immunohistochemistry experiment, we found expression of EAAT2 in the peripheral sensory fibres innervating the masseter muscles, confirming previous studies suggesting that it is the predominant excitatory amino acid transporter in the periphery[17,24].

The level of NGF-induced increase of SP in peripheral fibres was also in agreement with the NGF-induced SP increase in trigeminal ganglion neurons innervating the masseter muscles from an earlier study[11]. Although our results suggest that NGF induced glutamate expression in peripheral sensory fibres, the PGP9.5-positive neuronal structures may also include other neuronal tissues in the muscle that express glutamate. For example, a previous study found that rat masseter muscle spindle afferents express the glutamate vesicular transporter VGLUT1, suggesting glutamate release, while intramuscular injection of glutamate increased the jaw muscle stretch reflex in a human experiment[25,26]. This may suggest that NGF may also affect muscle proprioception through the peripheral glutamatergic system.

In the microdialysis experiment, the interstitial glutamate concentrations between PBS-injected and NGF-injected masseter muscles were not different. The interstitial glutamate concentrations observed in this study were consistent with those from an earlier study (~20 μM)[8]. By contrast, the immunohistochemistry results revealed elevated expression of glutamate in sensory nerve fibres in the NGF-injected masseter muscle. Intramuscular injection of botulinum neurotoxin, which blocks vesicular release of glutamate, reduced interstitial glutamate concentrations by about 30% in the temporalis muscle, which suggests that release of glutamate from the endings of muscle afferent fibres contributes to the concentration of glutamate measured in the interstitial fluid[27]. However, one possibility is that the increased glutamate visualised immunohistochemically is not being packaged into vesicles, but instead reflects increased metabolic activity within the axons induced by NGF treatment. On the other hand, it may simply be that a significant increase in glutamate occurred, but was not detected due to limitations of intramuscular microdialysis. The insertion of the microdialysis probe induces significant trauma to the surrounding muscle tissue, which results in a local inflammatory reaction around the probe insertion site. Inflammation-related extravasation of plasma may serve to reduce the concentration of glutamate around the probe. Plasma concentrations of glutamate are similar to those found in muscle tissue, so this would tend to bring the concentration of glutamate measured closer to the value in blood, due to dilution. A potential limitation of the study was that the immunohistochemistry experiment was not blinded. However, a consistent quantification method was vigorously followed to limit potential bias.

In this study, NGF increased glutamate expression in the sensory fibres innervating the masseter muscle. This result suggests that, in conjunction with increasing the expression of NMDA receptors on the fibres, NGF may induce a local region of increased glutamatergic tone in the muscle, which may result in a positive feedback cycle of glutamate – NMDA receptor sensitisation[12,13,14]. Our results may shed light on the mechanism of the tender regions observed in TMD patients with myofascial pain. It has been proposed that these are regions of micro-inflammation caused by repetitive trauma from activities such as excessive clenching and bruxing, and are associated with elevated levels of algogenic substances including glutamate, which is released by a variety of non-neuronal cells such as mast cells and macrophages[2,28,29]. The elevated glutamate expression and upregulation of NMDA receptor expression in masseter muscle mechano-nociceptors, mediated by NGF, may contribute to the pain and sensitisation in myofascial TMD patients[11].

Our results suggest that NGF induces mechanical sensitivity in the masseter muscle, in part by increasing glutamate expression in the sensory nerve endings innervating the muscle, and this effect is local only to the site of NGF injection.

Conflicts of interest

The authors reported no conflicts of interest related to this study.

Author contribution

Dr Hayes WONG designed the study, performed the experiments and wrote the manuscript; Dr Brian E. CAIRNS designed the study, wrote and revised the manuscript.

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