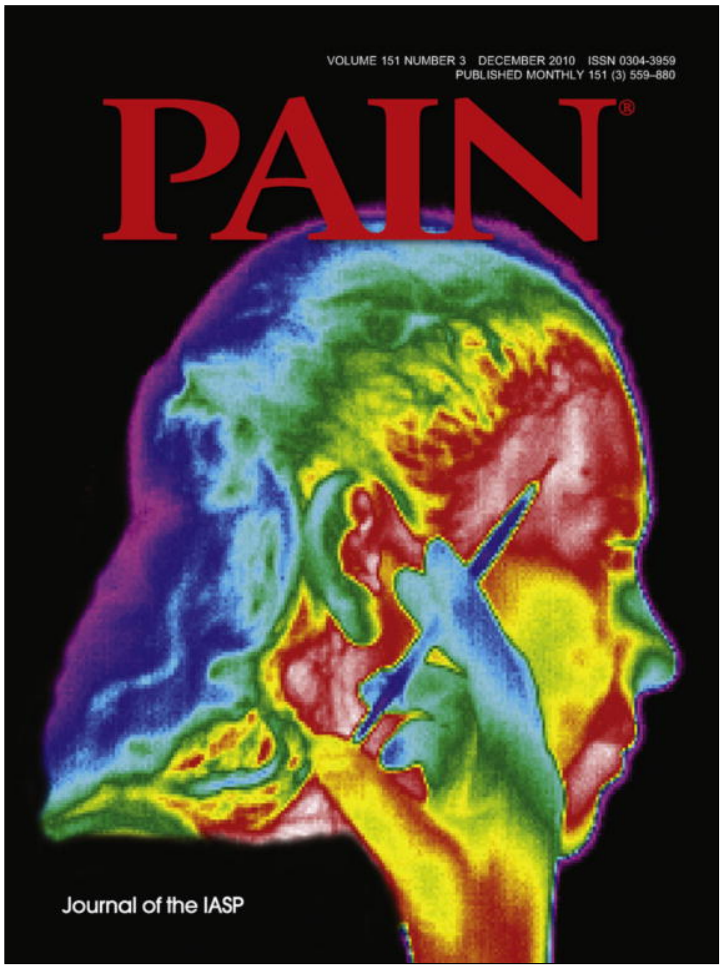

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Botulinum neurotoxin type A (BoNTA) decreases the mechanical sensitivity of nociceptors and inhibits neurogenic vasodilation in a craniofacial muscle targeted for migraine prophylaxis

Parisa Gazerani [a,b], Sammy Au [a], Xudong Dong [a], Ujendra Kumar [a], Lars Arendt-Nielsen [b], Brian E. Cairns [a,*]

[a] The Faculty of Pharmaceutical Sciences, The University of British Columbia, Vancouver, BC, Canada
[b] Center for Sensory-Motor Interaction (SMI), Dept. of Health Science and Technology, Aalborg University, Aalborg, Denmark

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ABSTRACT

The mechanism by which intramuscular injection of BoNTA into the craniofacial muscles decreases migraine headaches is not known. In a blinded study, the effect of BoNTA on the mechanical and chemical responsiveness of individual temporalis muscle nociceptors and muscle neurogenic vasodilation was investigated in female rats. Mechanical threshold was measured for 3 h following intramuscular injection of BoNTA or vehicle, and for 10 min after a subsequent injection of the algogen glutamate. Injection of BoNTA significantly increased the mechanical threshold of muscle nociceptors without altering the muscle surface temperature and blocked glutamate-induced mechanical sensitization and neurogenic vasodilation. None of these effects were reproduced by pancuronium-induced muscle paralysis. Western blot analysis of temporalis muscles indicated that BoNTA significantly decreased SNAP-25. Measurement of interstitial glutamate concentration with a glutamate biosensor indicated that BoNTA significantly reduced glutamate concentrations. The mechanical sensitivity of muscle nociceptors is modulated by glutamate concentration through activation of peripheral NMDA receptors. Immunohistochemical experiments were conducted and they indicated that half of the NMDA-expressing temporalis nerve fibers co-expressed substance P or CGRP. Additional electrophysiology experiments examined the effect of antagonists for NMDA, CGRP and NK1 receptors on glutamate-induced effects. Glutamate-induced mechanical sensitization was only blocked by the NMDA receptor antagonist, but muscle neurogenic vasodilation was attenuated by NMDA or CGRP receptor antagonists. These data suggest that injection of BoNTA into craniofacial muscles acts to decrease migraine headaches by rapidly decreasing the mechanical sensitivity of temporalis muscle nociceptors through inhibition of glutamate release and by attenuating the provoked release of CGRP from muscle nociceptors.

1. Introduction

The most common forms of primary headaches (tension type, migraine and chronic daily headache) affect more than 50% of the general population with a higher incidence in women than in men [61]. The disability and health effects associated with frequent episodes of headaches highlight the value of preventive pharmacotherapy, which aims to reduce the frequency, severity, duration and disability. N-Methyl-D-Aspartate (NMDA) receptor antagonists, gap-junction blockers (e.g. tonabersat) and angiotensinii type 1 blockers (e.g. candesartan) are currently being considered as potential novel prophylactic agents [26]. Botulinum neurotoxin type A (BoNTA) has also been proposed as an option for the prophylactic therapy of chronic headaches due to its low incidence of adverse effects [25,5,6,26]. However, clinical trials utilizing BoNTA as a preventive therapy have yielded inconsistent results as to the efficacy of this agent [58,5,12]. In part, this reflects the inherent difficulties in study design such as defining different subpopulations of headache sufferers and trial end points. This has fueled controversy as to the potential mechanism(s) of BoNTA when used for headache prophylaxis or treatment.

BoNTA is a toxin produced by the bacterium *Clostridium botulinum* [59] and it inhibits the release of acetylcholine (ACh) at neuromuscular junctions through the cleavage of synaptosome-associated protein of 25 kDa (SNAP-25) leading to prolonged muscle paralysis [53,36]. The muscle paralysis induced by BoNTA, however, does not appear to consistently correlate with its ability to decrease muscle pain [4]. On the other hand, while muscle pain is a common complaint during acute episodes of migraine and tension-type headache, studies have failed to demonstrate the abnormalities of muscle physiology involved in the

* Corresponding author. Address: The Faculty of Pharmaceutical Sciences, The University of British Columbia, 2146 East Mall, Vancouver, Canada V6T 1Z3. Tel.: +1 604 822 7715; fax: +1 604 822 3035.
E-mail address: brcairns@interchange.ubc.ca (B.E. Cairns).

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pathophysiology of either disorder [55,38]. It has, therefore, been proposed that BoNTA could decrease the frequency and severity of headaches by inhibition of the release of other neuropeptides/neurotransmitters which are involved in pain induction or transmission [4,47,56,64,18,24]. However, an effect of BoNTA on the response properties of craniofacial muscle nociceptors such as the temporalis muscle into which it is injected for headache prophylaxis has not been demonstrated.

The present study was conducted to test if intramuscular injection of BoNTA into craniofacial muscles exerts a direct effect on muscle nociceptors that could contribute to the ability of this toxin to decrease migraine occurrence. Accordingly, we tested two hypotheses: 1) injection of BoNTA into the temporalis muscle of female rats alters the response properties of temporalis muscle nociceptors to mechanical and noxious chemical (glutamate) stimuli. 2) BoNTA increases muscle perfusion, reflected as elevations in temporalis muscle surface temperature, to reduce ischemia-related muscle nociceptor sensitization.

2. Methods

2.1. Animals

Experiments to assess the effect of BoNTA on individual temporalis muscle nociceptors were conducted on anesthetized, adult female Sprague–Dawley rats (Charles River, Montreal, QC, Canada). Only one nociceptor was recorded in each rat. Rats were housed two per cage covered with soft bedding and food and water were freely available. The experimental protocol was approved by the University of British Columbia Animal Care Committee and the study was performed in accordance with the guidelines established by the Canadian Council on Animal Care and the International Association for the Study of Pain for the use of laboratory animals in research. Experiments were performed in a semi-dark lab with relative constant temperature (20.2 ± 0.7 °C) and humidity (36 ± 6%).

2.2. Anesthesia and surgical preparation

Rats were anaesthetized with isoflurane (AErrane, Baxter Corporation, Mississauga, ON, Canada; 2–2.5%) and oxygen (97–98%). A catheter was inserted into the carotid artery to monitor the blood pressure. A tracheal tube was inserted into the trachea to permit artificial respiration. Body temperature was measured with a rectal thermometer and maintained at 37.0 ± 0.2 °C with an electric heating pad. The heart rate, blood pressure and expired CO_2 were monitored throughout the experiment.

The animals were positioned prone in a stereotaxic frame; the skin over the dorsal surface of the skull was reflected and a small trephination in the skull bone was made to allow lowering of a microelectrode through the brain into the trigeminal ganglion for recording. The skin and muscle overlying the neck were gently dissected to expose the brainstem and the dura overlying the brain-

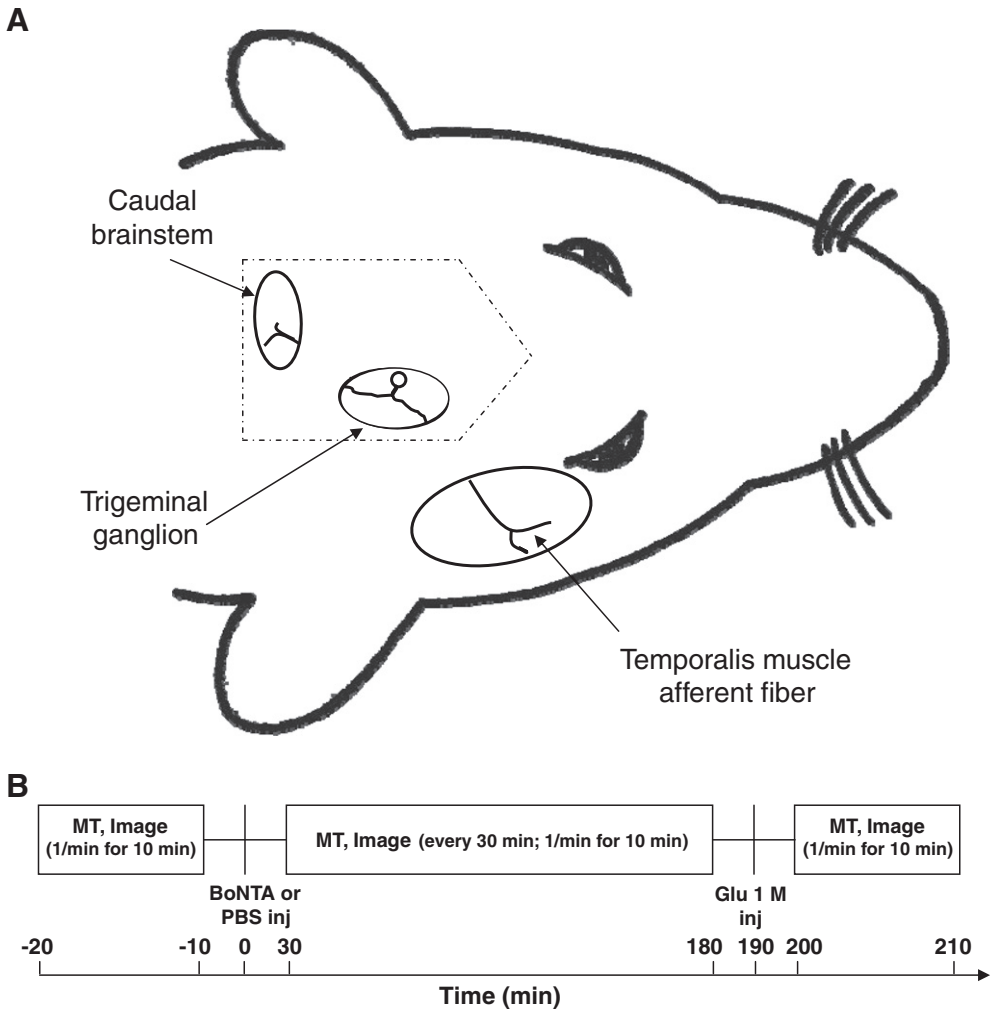

Fig. 1. (A) The location of the caudal brain stem, trigeminal ganglion and temporalis muscle are shown on the schematic drawing of the rat's head. (B) The experimental protocol is shown. The numbers represent time in minutes. (MT: Mechanical threshold).

stem was removed to permit a stimulating electrode to contact the caudal brainstem (Fig. 1A).

Microscopic examination of epidermal cells from a vaginal lavage was performed to determine estrus stage prior to each experiment. Animals were euthanized at the end of experiments (100 mg/kg; Nembutal, Abbott Laboratories, Chicago, IL, USA).

2.3. Stimulation and recording

The activity of individual temporalis muscle nociceptors of the female rats was recorded from the trigeminal ganglion with a parylene-coated tungsten microelectrode (0.10", 2 M, A-M Systems Inc., Carlsborg, WA, USA). A fine-tipped cotton swab was applied to the temporalis muscle region as a mechanical search stimulus while the electrode was slowly lowered to identify afferent discharge in response to mechanical stimulation of the temporalis muscle. When an afferent fiber responded to mechanical stimulation, the skin overlying the temporalis muscle was pulled back and held with a forceps to expose the muscle for recording. To prevent drying, the surface of the muscle was covered with mineral oil.

In previous studies, it has been shown that the caudal brainstem is a key projection site for putative nociceptors that innervate craniofacial muscle [16,15,23]. Antidromic collision was used to confirm the projection of temporalis muscle afferent fibers to the caudal brainstem and thus identify them as putative nociceptors. To estimate the conduction velocity (CV), the distance between the stimulating and recording electrodes was divided by the latency of the antidromic action potential of each nociceptor [15].

2.4. Thermal imaging

The surface temperature of the exposed temporalis muscle was monitored with an infrared camera (ThermaCAM EX320, FLIR Systems AB, Danderyd, Sweden). The infrared camera was adjusted on a tripod at an angle suitable to capture the entire rat head including the exposed temporalis muscle surface. The distance between the lens and the surface of the temporalis muscle and the angle of the camera was kept identical for all recordings. A piece of thick black paper was used under the animal body to avoid the light reflection and improve resolution of the image from the background. The desired area for the temperature measurement was marked with a silver pen to be detectable in thermo images. A region of 5×5 mm with the injection point at the center was used for temperature analysis. The temperature resolution of this camera is 0.1 °C. Images were recorded on a hard drive for off-line analysis of the pattern and local temperature change.

2.5. BoNTA experimental design

Experiments to assess the effect of BoNTA on individual temporalis muscle nociceptors were conducted on 24 anesthetized, adult female Sprague–Dawley rats. In each experiment, baseline mechanical threshold (MT; minimum force required to evoke afferent discharge) was measured with an electronic von Frey hair (model 1601C, Life Science, Woodland Hills, CA, USA) and the tissue surface temperature was measured with an infrared camera for a total of 10 min with an interval of one minute between each recording (Fig. 1B).

After baseline recording, the tip of a 26 gauge needle, connected via a polyethylene tube to a 50 μl Hamilton syringe (Hamilton, Reno, NV, USA), was inserted near the mechanoreceptive field of the muscle nociceptor and was used to inject solutions (BoNTA 5 units, Sigma–Aldrich, MO, USA, or phosphate buffered saline (PBS); 10 μl) intramuscularly. Injections were randomly assigned and the investigator (PG) assessing nociceptor MT was unaware of the content of injections.

Ongoing nociceptor discharge and tissue surface temperature (at 1 min intervals) were recorded for 10 min prior to injection. Then, 10 μl of the solution was injected and nociceptor discharge and tissue surface temperature (at 1 min intervals) were again recorded for 10 min. The MTs and tissue surface temperature were reassessed every half an hour (10 min/epoch, 1 min intervals) for a total time of 3 h. Then, the effect of BoNTA compared with PBS on glutamate-induced nociceptor discharge, nociceptor MT and the tissue surface temperature was assessed. The tip of a 26 gauge needle, connected via a polyethylene tube to a 50 μl Hamilton syringe (Hamilton, Reno, NV, USA), was inserted near the muscle nociceptor and any spontaneous fiber activity prior to injection and tissue surface temperature was recorded for 10 min. Glutamate (monosodium salt of L-glutamate, Sigma–Aldrich, MO, USA; 1 M, 10 μl; pH 7.2–7.6) was then injected and any evoked activity and tissue surface temperature were recorded for 10 min. Afterwards, the MTs and thermo images were taken every minute for a total of 10 min (Fig. 1B).

2.6. Effect of muscle paralysis

To investigate whether any of the effects of BoNTA were a result of muscle paralysis, additional experiments (in 4 female rats) were undertaken to test the effect of the neuromuscular blocker pancuronium bromide (1 mg/kg) on MT and glutamate-induced mechanical sensitization and neurogenic vasodilation. In these experiments, following 10 min baseline recording of the MT and temporalis muscle surface temperature, pancuronium was injected intravenously (via a femoral vein catheter). Starting 2 min after injection of pancuronium, MT and temporalis muscle surface temperature were reassessed for 10 min. Then the tip of a 26 gauge needle, connected via a polyethylene tube to a 50 μl Hamilton syringe (Hamilton, Reno, NV, USA), was inserted into the temporalis muscle and baseline nociceptor discharge was monitored for 10 min, after which glutamate (1 M, 10 μl) was injected and nociceptor discharge and tissue surface temperature were recorded for 10 min. At the end of this recording period, MT and tissue surface temperature were reassessed for a period of 10 min.

2.7. Western blot analysis

SNAP-25 content in the temporalis muscles was determined by Western blot analysis. Muscle tissue from the BoNTA or PBS injection site (right temporalis muscle) and the intact contralateral site (left temporalis muscle) ($n = 5$ in each group) was used. An area of approximately 1 cm^2 for each muscle tissue sample was dissected after euthanasia and kept frozen ($\leqslant -20$ °C) until homogenization. Muscle samples were homogenized, centrifuged (11'000 rpm, 20 min at 4 °C) and the supernatant was collected. The Bradford method was used to determine the protein concentration (μg/μl) of the homogenate. Samples containing 50 μg protein were loaded onto a 5% stacking gel/12% SDS–Polyacrylamide running gel and electrophoresed. The proteins were then transferred onto Hybond ECL Nitrocellulose membranes (GE Healthcare, Buckinghamshire, UK). The membranes were blocked with 5% non-fat dried milk (BioRAD Laboratories, CA, USA) and incubated overnight at 4 °C with anti-SNAP-25 antibody produced in rabbits (Sigma–Aldrich, MO, USA) diluted 1:5000. SNAP-25 bands were visualized using enhanced chemiluminescence. GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) was used as a loading control.

2.8. Measurement of temporalis muscle interstitial glutamate concentration by glutamate biosensor

Glutamate biosensors (Pinnacle Technology, Inc., USA) were calibrated *in vitro* according to the manufacturer's instructions. The glutamate biosensors were then inserted into the temporalis

muscles bilaterally through an injection cannula which consisted of a 26 gauge needle connected via a polyethylene tube to a 50 μl Hamilton syringe to permit the injection of BoNTA or PBS. The tip of the needle was positioned exactly 2 mm from the tip of the glutamate biosensor. After insertion into the muscle, the probe was allowed to stabilize for at least 60 min prior to any experiments being undertaken. Once the probe's output was stabilized, a 10 min baseline was recorded, followed by the injection of BoNTA (5 units, Sigma–Aldrich, MO, USA; 10 μl) and PBS (10 μl) over 5 s. Glutamate concentration was recorded continuously (sampling rate 4 Hz) for 3 h after the injections.

2.9. Immunohistochemistry

The expression of NMDA receptor NR2B subunits and co-expression of CGRP and SP by nerve fibers innervating the temporalis muscle were assessed by fluorescence immunohistochemistry. Temporalis muscles of intact female rats ($n = 6$) were cut into 10 μm thick sections with a cryostat microtome. Every third section was selected for staining for a total of five sections per rat. Sections were incubated in 10% normal goat serum (NGS) and 0.2% Triton X-100 in PBS for 1 h at room temperature followed by overnight incubation with primary antibodies in 1% NGS. Nerve fibers were identified with the use of rabbit polyclonal antibody against rat PGP 9.5 (1:2000; Abcam Inc., Cambridge, MA). PGP 9.5 is a neuronal marker and serves to identify nerve fibers. NR2B subunits were identified with the use of mouse monoclonal antibody (1:400; Chemicon International Inc., Billerica, MA). CGRP was identified with the use of goat polyclonal antibody (1:200; Abcam Inc., Cambridge, MA) and SP was identified with the use of guinea pig polyclonal antibody (1:700; Abcam Inc., Cambridge, MA). CY3-conjugated goat anti-rabbit IgG (1:500; Jackson Immunoresearch Laboratories Inc., West Grove, PA) was used to visualize the PGP 9.5-immunoreactivity (IR); FITC-conjugated goat anti-mouse IgG (1:100; Jackson Immunoresearch) was used to visualize NR2B-IR; AMCA-conjugated donkey anti-goat IgG (1:100; Jackson Immunoresearch Laboratories Inc., West Grove, PA) was used to visualize the CGRP-IR; and AMCA-conjugated donkey anti-guinea pig IgG (1:100; Jackson Immunoresearch Laboratories Inc., West Grove, PA) was used to visualize the SP-IR. Sections were visualized under a Leica DML fluorescent microscope attached to a Cool Snap CCD camera. The specificity of receptor-like immunoreactivity was confirmed by omission of the primary antibodies, and in the case of SP and CGRP by preadsorption of the primary antibody with the appropriate antigen.

2.10. Co-administration of glutamate, CGRP and SP antagonists

To study the role of possible mediators of glutamate-evoked mechanical sensitization and neurogenic vasodilation, additional nociceptor recordings were performed where glutamate ($n = 6$, 1 M) was injected alone or in combination with the CGRP antagonist CGRP 8–37 ($n = 6$, 20 μM; Tocris bioscience, Bristol, UK), the SP antagonist L-703,606 ($n = 6$, 2.5 μM; Sigma–Aldrich, Oakville, ON, Canada), or the competitive NMDA receptor antagonist 2-amino-5-phosphonopentanoic acid (APV) ($n = 6$, 20 mM; Sigma–Aldrich, MO, USA). In these experiments, following 10 min baseline recording of the MT and temporalis muscle surface temperature, the tip of a 26 gauge needle, connected via a polyethylene tube to a 50 μl Hamilton syringe (Hamilton, Reno, NV, USA), was inserted near the muscle nociceptor and any spontaneous fiber activity prior to injection and tissue surface temperature was recorded for 10 min. The solutions were then injected and any evoked activity and tissue surface temperature were recorded for 10 min. Afterwards, the MTs and thermo images were taken every minute for a total of half an hour.

2.11. Data analysis

2.11.1. BoNTA experiments

Sample size estimation using ANOVA suggested that samples of 12 nociceptors per group would allow the detection of a 25% difference in MT between PBS and BoNTA ($\alpha = 0.05$, power = 0.80). Therefore, in the initial BoNTA experiments, 12 nociceptors were assigned to each group.

The average MT from ten consecutive stimuli was used to determine the baseline and post injection MT (for each time epoch) for each fiber. Post injection MT was divided by the baseline MT to yield relative MT. Evoked discharge was calculated by subtracting the number of action potentials recorded during the 10 min epoch before injection from the number recorded during the 10 min epoch after injection.

A two-way repeated measures ANOVA was used on MT and tissue surface temperature data with time (repeated, within subject factor) and treatments (between subject factors) as factors to determine the main effect of each factor and interactions between factors. Holm-Sidak *post hoc* tests were used when a significant factor effect was revealed by ANOVA. An unpaired *t*-test was used for comparison between relative MT (before and after glutamate administration) in BoNTA- and PBS-treated groups. Glutamate-evoked activity of temporalis muscle nociceptors was compared between BoNTA- and PBS-treated groups using Mann–Whitney Rank Sum Test.

To analyze the thermo images, the desired area was marked by a silver pen prior to the recording (5 × 5 mm) and was labeled with the "area tool" of the analysis software (ThermaCAM Quick Report 1.0, FLIR Systems AB, Danderyd, Sweden). Then the average temperature (°C) of this area was calculated and used for the statistical tests. The rain palette was used for all analysis.

2.11.2. Pancuronium experiments

Paired *t*-tests were used to evaluate the effect of pancuronium on MTs and glutamate-evoked mechanical sensitization. A repeated measures ANOVA was used to evaluate the effect of various treatments on muscle surface temperature.

2.11.3. Quantitative Western blots

SNAP-25 was quantified by densitometric analysis using GAPDH as a loading control. The relative intensity of SNAP-25 band to the housekeeping protein GAPDH was then calculated and used for statistical analysis. The SNAP-25 content of the temporalis muscle in BoNTA and PBS-treated groups was compared by an unpaired *t*-test.

2.11.4. Interstitial glutamate concentration

Post injection interstitial concentration of glutamate (10 min each 30 min for 3 h) in each temporalis muscle was divided by pre injection interstitial concentration to yield relative glutamate concentration. A two-way repeated measures ANOVA with time and treatment as factors was used to assess differences between BoNTA and PBS on glutamate concentration in the temporalis muscle.

2.11.5. Immunohistochemistry

Fibers were considered positive when the intensity of the CY3, FITC and AMCA signals, respectively, exceeded the 95% confidence interval of the mean background intensity. The percentage of PGP 9.5 positive fibers that expressed NR2B, CGRP and substance P and the percentage of co-expression (NR2B & CGRP or NR2B & substance P) were calculated from the results obtained in 5 muscle tissue sections per rat. The percentages from all 5 rats were used to calculate the mean and standard error.

2.11.6. Antagonist experiments

Kruskal–Wallis one way ANOVA on ranks was used to assess changes in MTs and a one way repeated measures ANOVA was used on tissue surface temperature for the comparison of glutamate alone and in combination with antagonists (CGRP 8–37, L-703,606 and APV). Dunnett's *post hoc* test further was used to determine which antagonists affected glutamate-induced mechanical sensitization or glutamate-evoked neurogenic vasodilation.

2.11.7. Analysis software

The analysis software consisted of Spike2 (Cambridge Electronic Design, Cambridge, UK), SigmaStat version 3.0 (SPSS Inc., Chicago, IL, USA) SigmaPlot version 10.0 (Systat Software, Cranes Software International, Kamataka, India) and SPSS version 16 (SPSS Inc., Chicago, IL, USA). Data in the text are reported as mean ± SEM (standard error of the mean). For all statistical tests, $P < 0.05$ was considered significant.

3. Results

3.1. Effect of BoNTA treatment

There were no significant differences in the CV, baseline MT, or estrous cycle stage between the BoNTA and the PBS groups. Injection of BoNTA did not evoke significantly more activity than PBS. However, injection of BoNTA significantly increased MT compared to PBS at 2.5 and 3 h post injection (Fig. 2A) without significantly modifying muscle surface temperature (Fig. 2B).

Intramuscular injection of glutamate (1 M) has been shown to reliably evoke discharge from craniofacial muscle nociceptors and mechanically sensitize them [15,13]. Glutamate injections evoked discharge from all 24 nociceptors, which confirmed that their terminations were within the temporalis muscle, although there was no significant difference in glutamate-evoked discharge when the BoNTA and PBS groups were compared (Fig. 3A). Glutamate-induced mechanical sensitization, however, was significantly attenuated in the BoNTA group when compared with the PBS group (Fig. 3B).

Intramuscular injection of glutamate has also been demonstrated to significantly increase blood flow through masticatory muscle [14]. Measurement of muscle surface temperature by thermographic imaging is a non-invasive means to assess changes in tissue perfusion. Typical thermo images taken from one female rat in the PBS-treated group are depicted in Fig. 4A. The images show that injection of glutamate after PBS significantly increased muscle surface temperature (2.5 °C); an indirect measure of muscle perfusion. In contrast, muscle surface temperature did not change if glutamate was injected after BoNTA (Fig. 4B).

3.2. Effect of Pancuronium

To rule out the possibility that the effect of BoNTA was due to muscle paralysis, additional experiments on 4 nociceptors recorded in 4 female rats were conducted to examine the effect of intravenous administration of a paralytic dose of pancuronium. Pre injection of pancuronium had no significant effect on nociceptor MT, did not inhibit glutamate-induced mechanical sensitization (Fig. 5A) or alter glutamate-induced increases in temporalis muscle surface temperature (Fig. 5B).

3.3. Effect of BoNTA on SNAP-25

BoNTA is thought to exert its effects through degradation of the vesicular docking protein SNAP-25. In Western blot analysis, a band at 25 kDa, the expected size of SNAP-25, was observed as shown in Fig. 6A. SNAP-25 content was significantly decreased in BoNTA-treated temporalis muscles (Fig. 6B).

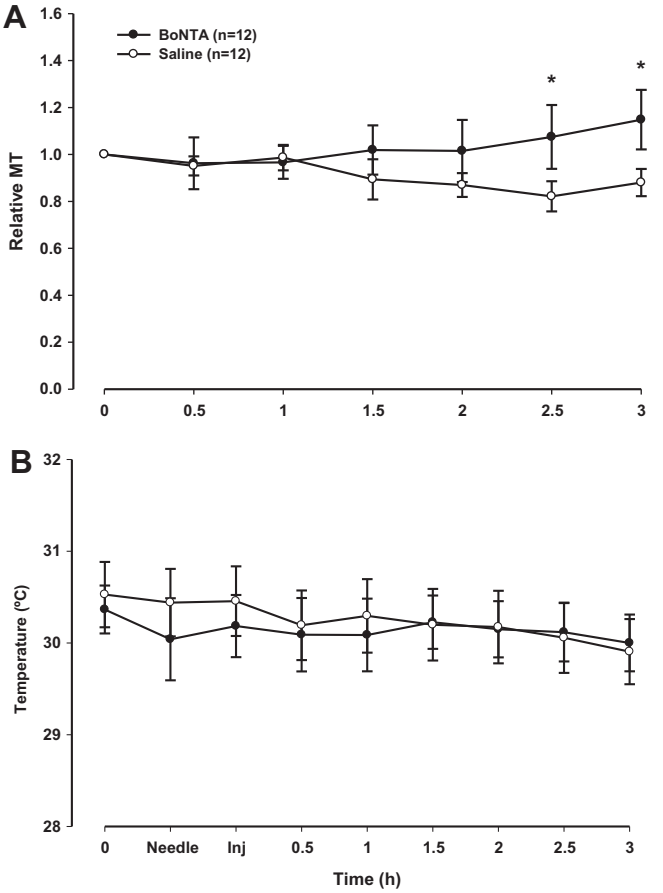

Fig. 2. (A) The line and scatter plot illustrates the mean (error bars: SEM) relative (change from baseline) MT of female temporalis muscle nociceptors following intramuscular administration of BoNTA and PBS ($n = 12$ in each group). Two-way repeated measures ANOVA revealed a significant effect of treatment but not time on the muscle nociceptor MTs following intramuscular injection of BoNTA and PBS ($F_{1,154} = 4.401$, $P = 0.048$). There was also an interaction between treatment and time ($F_{7,154} = 5.302$, $P < 0.001$). A Holm-Sidak *post hoc* test indicated that average relative MTs in BoNTA-treated group were significantly greater (*) than those in the PBS -treated group at 2.5 ($P = 0.033$) and 3 ($P = 0.024$) hours following treatment. (MT: Mechanical threshold). (B) The line and scatter plot illustrates the mean (error bars: SEM) temperature change (°C; 3 h) following intramuscular injection of BoNTA and PBS into the female rats' temporalis muscle. BoNTA had no significant effect on the muscle surface temperature over the 3 h recording period.

3.4. Effect of BoNTA on Interstitial Glutamate Concentration

The mechanical sensitivity of craniofacial muscle nociceptors can be modulated by alterations in interstitial glutamate concentration [13]. Glutamate selective biosensors were employed to determine whether BoNTA might be altering the release of glutamate into the interstitial space of the temporalis muscle. There was no significant difference in the mean interstitial concentration of glutamate when the left (17 ± 4 μM; control) and right (20 ± 3 μM; BoNTA) temporalis muscles were compared. Injection of PBS into the temporalis muscle resulted in a slow decline in glutamate concentrations. Following the intramuscular administration of BoNTA, interstitial glutamate declined more rapidly and to a greater extent than following PBS administration (Fig. 7).

3.5. Expression of CGRP and SP in Temporalis Nerve Fibers

Immunohistochemistry was undertaken to demonstrate whether NR2B subunit positive nerve fibers within the temporalis

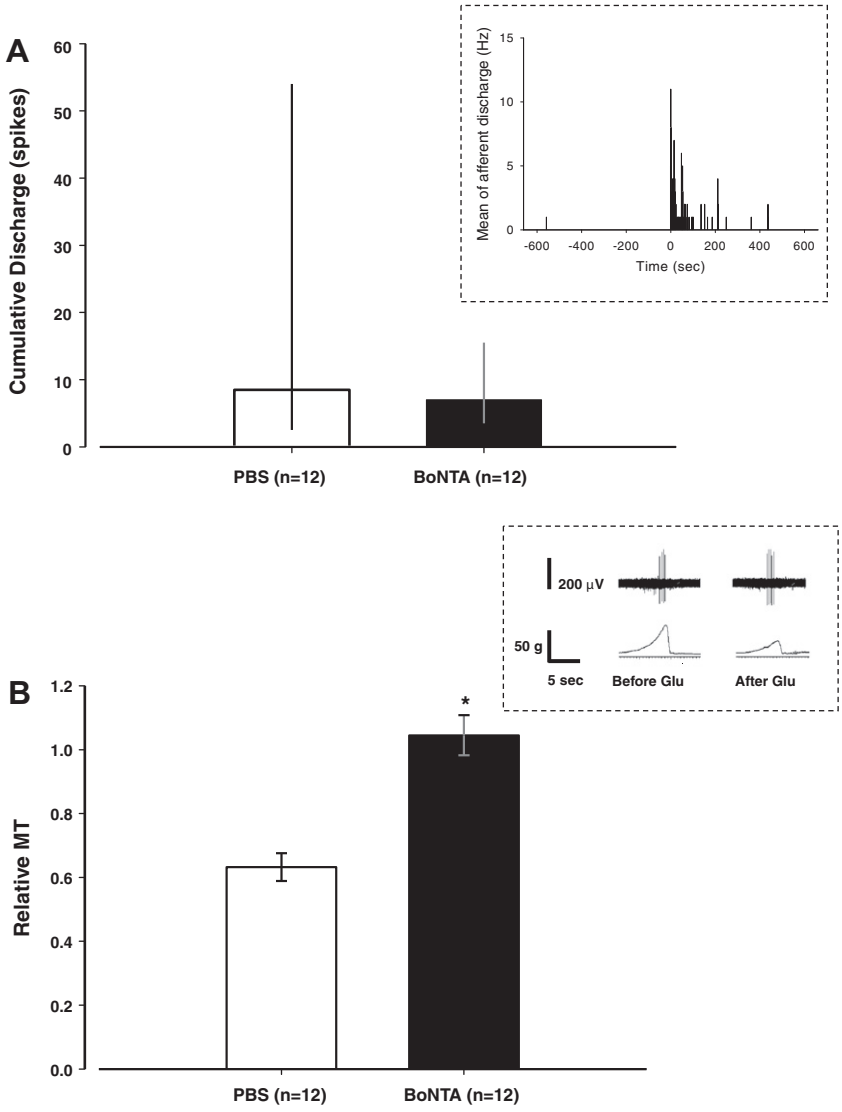

Fig. 3. (A) The vertical bar graph shows the median (error bars: interquartile range) cumulative nociceptor discharge after injection of glutamate. BoNTA did not alter median glutamate-evoked temporalis nociceptor discharge ($n = 12$ in each group; Mann–Whitney Rank Sum Test, $P = 0.665$). The inset box shows an example of nociceptor (CV: 6.1 m/s) discharge evoked by injection of 1 M glutamate into the temporalis muscle. (B) The vertical bar graph shows the mean (error bars: SEM) relative MT after injection of glutamate. BoNTA significantly attenuated the ability of glutamate injections to decrease nociceptor MT (*: Unpaired *t*-test, $P < 0.05$). The box shows an example of nociceptor discharges (upper traces) in response to electronic von Frey hair mechanical stimuli (lower traces) before and after glutamate injection.

muscle express either CGRP or SP that could be released by glutamate injections. Approximately 70% of temporalis muscle nerve fibers expressed the NR2B subunit of NMDA receptor (Fig. 8). Roughly 50% of NMDA receptor-expressing nerve fibers co-expressed SP or CGRP.

3.6. Blocking of glutamate-induced mechanical sensitization and neurogenic vasodilation

The inhibition of glutamate-induced nociceptor mechanical sensitization by BoNTA suggested that a neuromodulatory substance other than glutamate might also be contributing to the observed effects of BoNTA on nociceptor MT. Our demonstration of co-localization of substance P and CGRP with NMDA receptors on nerve fibers in the temporalis muscle suggested that one or both of these neuropeptides could be released to mediate the mechanical sensitization. However, glutamate-induced mechanical sensitization was blocked only following the administration of the NMDA receptor antagonist APV; CGRP and NK1 receptor antagonists had no effect on glutamate-induced mechanical sensitization (Fig. 9A). Glutamate-induced increases in muscle surface temperature, an indirect measure of neurogenic vasodilation, were, however, significantly blocked following the administration of antagonists for CGRP and NMDA receptors, but not NK1 receptors (Fig. 9B).

4. Discussion

Prophylactic use of BoNTA to reduce migraine has yielded inconsistent results [6,50]. The largest and most recent parallel studies conducted on patients with chronic migraine treated with BoNTA demonstrated significant reduction of headache days [12]. Recent studies also show that patients with imploding and ocular migraines are more responsive to BoNTA than those with exploding migraines [41,10].

For migraine prophylaxis, BoNTA is injected into uninjured craniofacial muscles [22] similar to the approach used in the present study. Previous animal studies have used inflammation and/or

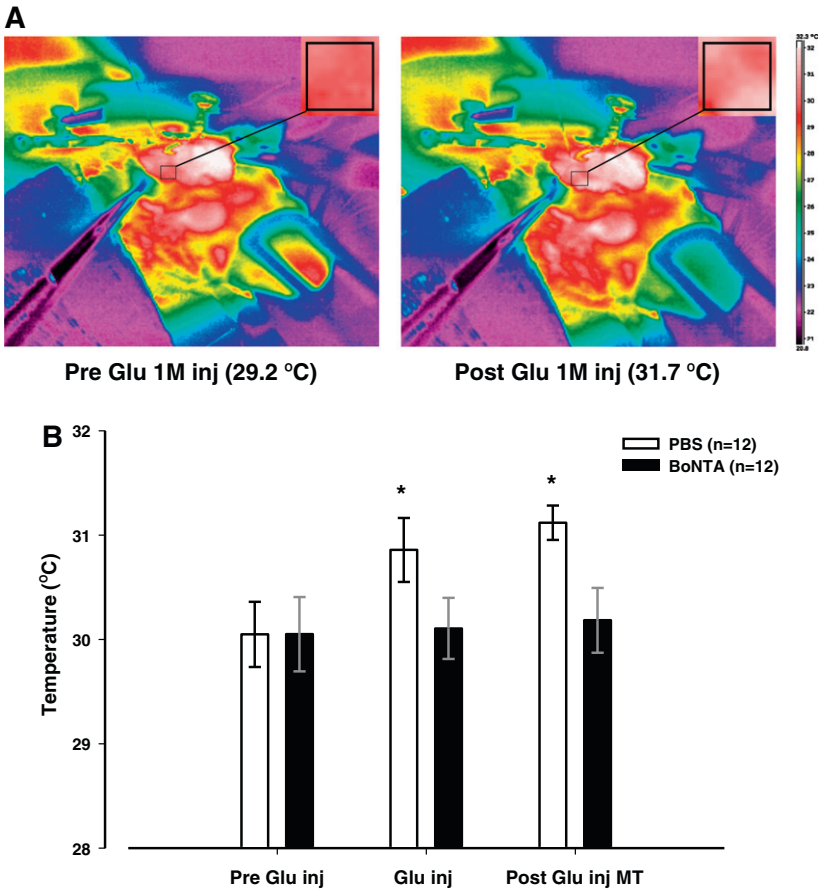

Fig. 4. (A) Typical thermo images from the temporalis muscle showing the temperature before and following the intramuscular administration of glutamate (1 M) in a PBS-treated female rat. (B) The vertical bar graph shows the mean (error bars:±SEM) surface temperature of the temporalis muscle pre glutamate injection, post glutamate injection and following the MT measurement in BoNTA- and PBS-treated groups ($n = 12$ in each group). *indicates the difference from pre glutamate injection in PBS-treated group (Two-way repeated measures ANOVA, $P < 0.05$).

neuropathic injury to investigate the analgesic mechanism of BoNTA and have reported that BoNTA decreases nocifensive behavior, mechanical sensitization and inflammation of the rat hind paw [52,45,21]. These analgesic and anti-inflammatory effects were not observed for several hours to several days after injection of BoNTA. In humans, injection of BoNTA has also been shown to reduce both cutaneous mechanical sensitization and neurogenic inflammation induced by intradermal injection of capsaicin [32,31]. It is then suggested that BoNTA can exert anti-inflammatory actions through inhibition of neurogenic vasodilation that may contribute to its analgesic effects in inflammatory pain models. In the present study, BoNTA rapidly (within 2.5 h) increased the MT of rat temporalis muscle nociceptors without significantly altering muscle blood flow, as reflected by a lack of change in muscle surface temperature. This finding indicates that the desensitizing effect of BoNTA on muscle nociceptors does not result from a decrease in muscle blood flow.

The analgesic effect of BoNTA in the formalin model has been suggested to be related to a blockade of vesicular glutamate release in the skin after BoNTA treatment [21,51]. The mechanical sensitivity of craniofacial muscle nociceptors in rats also appears to be regulated, in part, by interstitial glutamate concentrations. Injection of glutamate or glutamate receptor subtype selective agonists into masticatory muscles briefly excites (~ 1–5 min of discharge) and induces a prolonged (~ 3 h) mechanical sensitization of muscle nociceptors through activation of peripheral glutamate receptors [15,17,20,44,46]. The interstitial concentration of glutamate in masticatory muscles is ~ 20 μM, and a 2–3 times increase in this concentration has been shown to induce nociceptor mechanical sensitization through peripheral NMDA receptor activation [13]. It has been speculated that vesicular release of glutamate by nerve fibers makes a significant contribution towards the resting interstitial concentration of glutamate in tissues [15,13,19]. In the present study, BoNTA was found to significantly reduce the levels of the vesicular docking protein SNAP-25 after injection into the temporalis muscle. BoNTA also significantly reduced baseline interstitial glutamate concentration in the temporalis muscle by ~70% after 2 h. Taken together, these data suggest that BoNTA blocks the vesicular release of glutamate in the rat temporalis muscle. However, the decrease in interstitial glutamate concentration appeared to precede rather than coincide with the elevation in nociceptor MT. This time lag between changes in glutamate interstitial concentration and nociceptor MT in combination with our finding that BoNTA inhibited mechanical sensitization induced by a subsequent injection of glutamate into the temporalis muscle, suggested that inhibition of the release of a neuromodulatory substance other than glutamate might also be contributing to the observed effects of BoNTA on nociceptor MT.

SP and CGRP have been suggested to play an important role in peripheral sensitization and neurogenic inflammation in craniofacial muscles that may also be relevant to the initiation of headaches [54,27]. Trigeminal ganglion neurons that project to masticatory muscle express both SP and CGRP-like immunoreactivity [3]. The CGRP antagonist CGRP 8–37 has been shown to attenuate mechanical sensitization and plasma protein extravasation in the masseter muscle produced by intramuscular injection

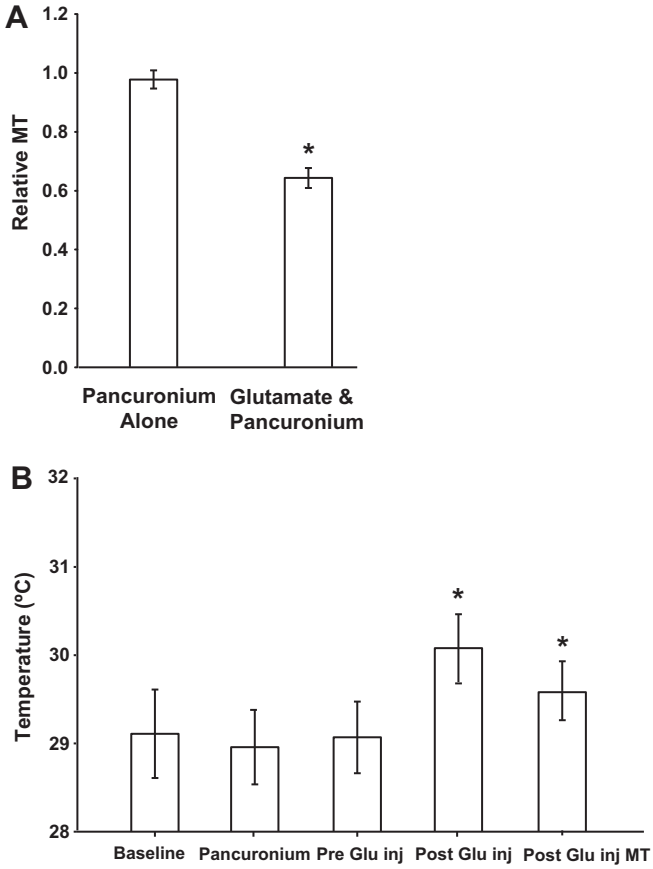

Fig. 5. (A) The vertical bar graph indicates the mean (error bars:±SEM) relative change in nociceptor MT after intravenous injection of pancuronium (1 mg/kg) and the effect of a subsequent injection of glutamate (1 M) in 4 female rats. Note that pancuronium did not alter the MT relative to baseline, while glutamate substantially reduced the MT (*: Paired *t*-test, $P < 0.05$). These results indicate that muscle paralysis was not responsible for the effects of BoNTA on MT. (B) The vertical bar graph illustrates the mean (error bars:±SEM) surface temperature of the temporalis muscle measured at baseline, immediately after pancuronium injection, just prior to glutamate injection (pre glu inj), just post glutamate injection (post glu inj) and during the assessment of MT, 10 min post glutamate injection (post glu MT). Injection of pancuronium did not affect muscle surface temperature, and did not alter the ability of glutamate injection into the temporalis muscle to increase muscle surface temperature. $^{*}P < 0.05$ one-way repeated measures ANOVA.

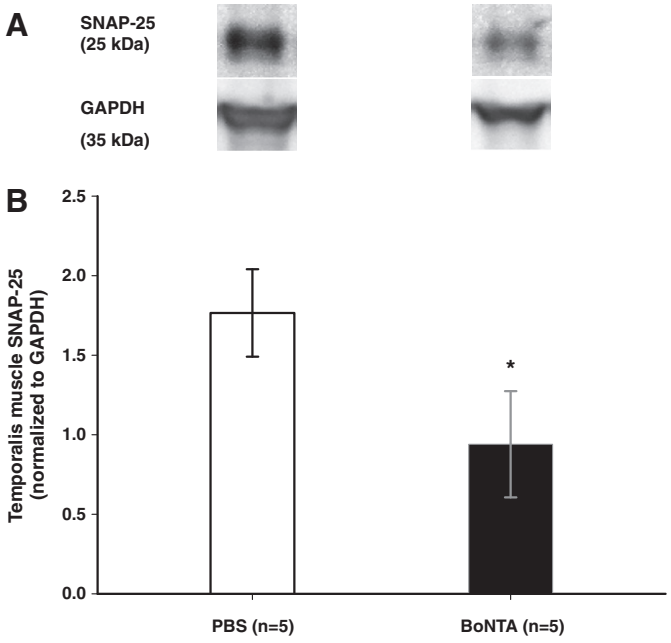

Fig. 6. (A) Representative gels showing expression of SNAP-25 and GADPH in PBS (left) and BoNTA (right) treated temporalis muscles. (B) The vertical bar graph illustrates mean (error bars:±SEM) SNAP-25 content (normalized to GAPDH) of female temporalis muscle in BoNTA- and PBS-treated groups ($n = 5$ in each group). BoNTA significantly reduced SNAP-25 levels compared to PBS (*: Unpaired *t*-test, $P = 0.038$). SNAP-25: Synaptosome-associated protein of 25000 daltons; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

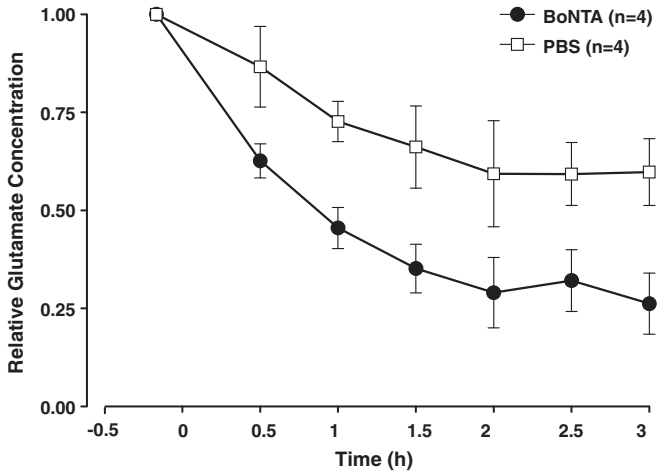

Fig. 7. The line and scatter plot illustrates the mean (error bars: SEM) relative change in glutamate concentration after injection of BoNTA and PBS into the right and left temporalis muscles, respectively ($n = 4$). Glutamate concentrations declined more rapidly and to a greater extent in temporalis muscle treated with BoNTA than PBS (Two-way repeated measures ANOVA, $P < 0.05$).

of Freund's complete adjuvant [3]. Injection of SP into the masseter muscle did induce edema formation, however, NK1 antagonists were not found to be effective inhibitors of neurogenic inflammation in this muscle [57]. In the present study, glutamate, which is not inflammatory [30], was used to induce mechanical sensitization and increase muscle blood flow in the temporalis muscle. It was found that NMDA-expressing nerve fibers in temporalis muscle, which could be excited by glutamate, co-expressed both CGRP and SP. Injection of glutamate did appear to cause the release of both neuropeptides, as glutamate-induced neurogenic vasodilation was attenuated by CGRP and, to a lesser extent, NK1 receptor antagonists. However, despite evidence of their release after intramuscular injection of glutamate, neither CGRP nor SP appeared to contribute significantly to glutamate-induced mechanical sensitization of temporalis muscle nociceptors. As both NMDA receptor antagonists and BoNTA did significantly inhibit glutamate-induced mechanical sensitization, one explanation for this finding is that a single intramuscular injection of a high concentration of glutamate is sufficient to induce a prolonged depolarization of the temporalis nociceptor terminals which results in the continued vesicular release of glutamate itself; an effect which would be blocked by BoNTA. It is equally feasible that an as yet unidentified substance is released by elevated interstitial glutamate concentrations to mediate glutamate-induced mechanical sensitization. Although the mechanism is not entirely resolved, the results of the present study do indicate that attenuation of vesicular release by BoNTA increases the MT of temporalis muscle nociceptors *in vivo*.

CGRP is proposed to play a significant role in migraine [60,63]. A previous study reported that BoNTA did not alter basal CGRP release but dramatically reduced the evoked CGRP release in *in vitro* models [24]. In the present study, it was also found that BoNTA had no effect on resting tissue perfusion in the temporalis

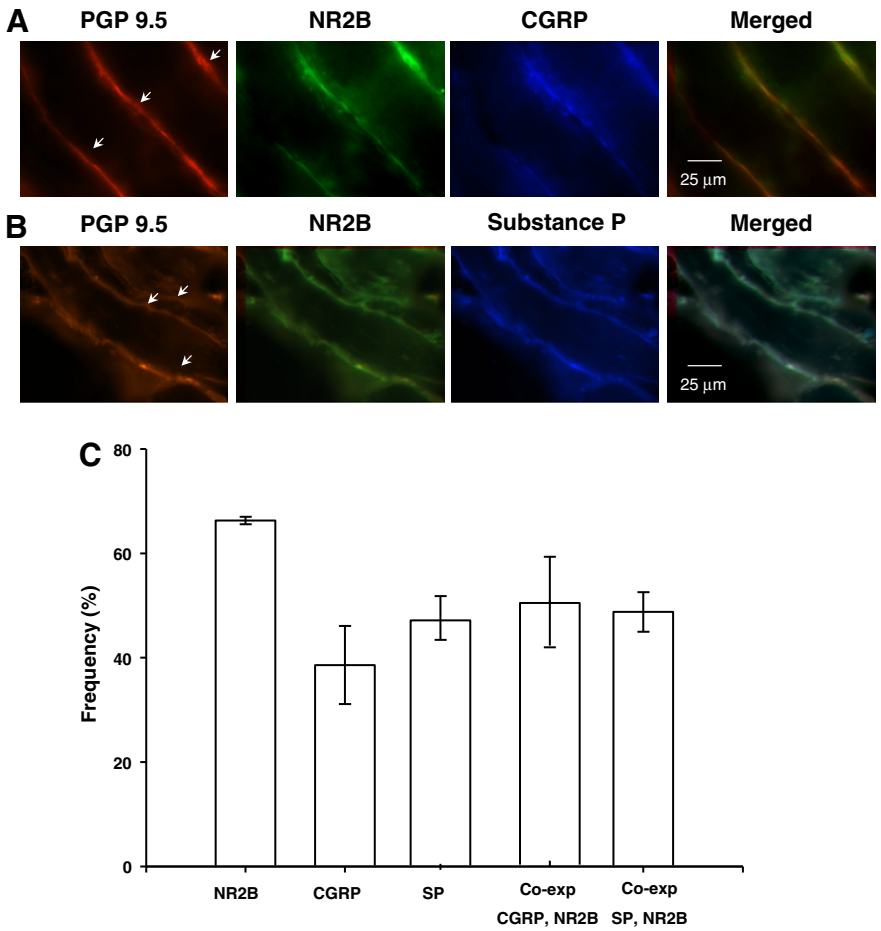

Fig. 8. (A) Example photomicrographs show temporalis muscle nerve fibers (PGP 9.5 positive – indicated by white arrows) that co-expressed NR2B subunits and CGRP. (B) Example photomicrographs show temporalis muscle nerve fibers (PGP 9.5 positive – indicated by white arrows) that co-expressed NR2B subunits and SP. (C) To the left, the bar graph shows the mean (error bars:±SEM) expression of NR2B, CGRP, and SP in temporalis nerve fibers. To the right, the mean (error bars:±SEM) co-expression of CGRP and SP in NR2B-positive fibers, respectively, is shown. PGP 9.5: Protein gene product 9.5; NR2B: NMDA receptor subunit; CGRP: calcitonin gene related peptide; SP: Substance P).

muscle but did inhibit neurogenic vasodilation evoked by injection of glutamate. The vesicular release of CGRP mediates neurogenic vasodilation evoked by glutamate based on our findings that temporalis nerve fibers express CGRP and glutamate-evoked vasodilation was inhibited by a CGRP receptor antagonist. Therefore, BoNTA could mediate its migraine prophylactic effects, in part, through inhibition of CGRP release, although future studies are required to test this concept.

4.1. Clinical implications of the present findings for migraine prophylaxis

There is controversy in the literature with regard to whether migraine and/or other headaches can be provoked by input from the craniofacial muscles. For example, while the EMG activity of craniofacial muscles (e.g. frontalis and temporalis muscles) do not significantly differ in migraine patients compared with healthy controls [39,8], removal of the corrugator supercilii muscle, a muscle on the forehead near the eye, can significantly improve migraine headaches in patients [35]. Migraine patients often complain of mechanical sensitivity of the craniofacial region with cutaneous allodynia developing in 79% of migraine patients during the headache [9,11]. While the pathogenesis of migraine remains incompletely understood, migraine-related mechanical sensitivity likely reflects both local changes in peripheral nociceptor excitability and central sensitization, which can lead to spreading of the allodynia outside of the craniofacial region [9,11]. Animal research indicates that certain cervical spinal cord and trigeminal nociceptive neurons receive input from both the dura and craniofacial muscles, and that ongoing nociceptive input from craniofacial muscle is sufficient to induce prolonged sensitization of nociceptive neurons [48,2,42,49], so it is feasible that this mechanism increases the potential for headache development.

Increased trigeminal nerve terminal excitability associated with peripheral sensitization may be accompanied by the release of vasoactive substances like glutamate, CGRP and SP in migraine [33,34,37]. In particular, an association between increased glutamate release and migraine is suggested by several studies where the concentration of glutamate in the blood was found to be significantly elevated in migraine sufferers compared to healthy controls and further elevated in migraine sufferers during attacks [29,1,28]. In healthy individuals, oral administration of monosodium glutamate (MSG), which increased blood levels 3–5 times above baseline, significantly increased reports of headache and pericranial muscle tenderness [7]. An ~70% reduction in blood glutamate concentrations reduced the frequency of migraine by half in a recent study [28]. Our findings suggest that injection of BoNTA into craniofacial muscles decreases interstitial glutamate concentration in the muscle and that this action was associated with an increase in nociceptor MT. BoNTA also prevented exogenously administered glutamate from inducing mechanical sensitization of muscle nociceptors. Based on these results, we conclude that injections

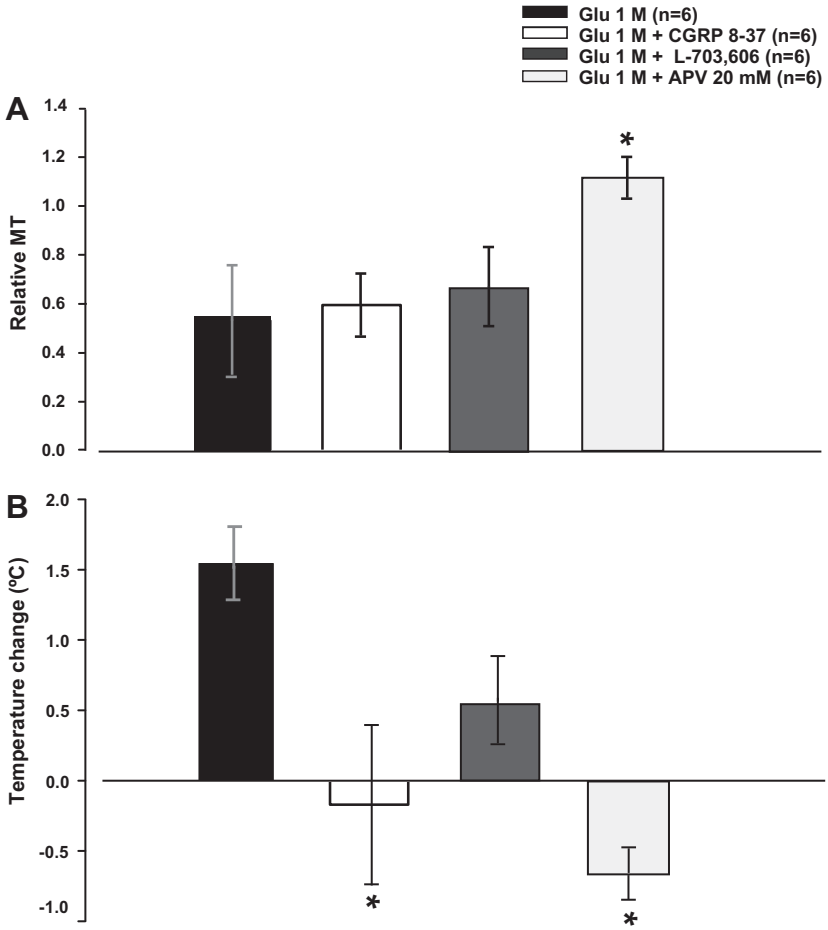

Fig. 9. (A) The bar graph shows the mean (error bars:±SEM) relative MT after injection of glutamate alone or co-administered with the CGRP receptor antagonist CGRP 8–37 (20 μM), the SP antagonist L-703,606 (2.5 μM) or the NMDA receptor antagonist APV (20 mM) ($n = 6$ in each group). Glutamate-induced decreases in MT were significantly attenuated by APV (Kruskal–Wallis one way ANOVA on Ranks followed by Dunnett's *post hoc*, $P = 0.027$) (MT: Mechanical threshold; Glu: Gluamate; CGRP: calcitonin gene related peptide; SP: Substance P; NMDA: N-Methyl-D-Aspartate; APV: 2-amino-5-phosphonopentanoic acid). (B) The bar graph indicates the mean (error bars:±SEM) temperature change (°C, from baseline) at the injection site in the temporalis muscle following intramuscular administration of glutamate alone or co-administered with the antagonists. Antagonism of either the NMDA or CGRP receptor resulted in complete attenuation of glutamate-induced increased muscle surface temperature. *ANOVA followed by Holm-Sidak *post hoc* test, $P < 0.001$ compared with Glu 1 M. (CGRP: calcitonin gene related peptide; NMDA: N-Methyl-D-Aspartate).

of BoNTA into craniofacial muscles of migraine sufferers reduce the local sensitivity of muscle nociceptors at the injection sites and speculate that BoNTA could also act to reduce blood glutamate concentration similar to other prophylactic treatments [28].

While there is little doubt that CGRP plays an important role in the pathogenesis of migraine, there is some controversy as to whether the levels of this vasoactive peptide are elevated in migraine sufferers [40,62,63]. However, intravenous administration of CGRP causes migraine-like headaches in migraine sufferers and it has been found that drugs which reduce the release of CGRP (e.g. sumatriptan) or block its receptors (e.g. tecalgepant) are effective migraine treatments and may also be effective for prophylaxis against migraine attacks [43,63]. In the present study, CGRP release was induced by elevated interstitial glutamate concentrations in the temporalis muscle and attenuated by injection of BoNTA. This finding suggests that the mechanism of BoNTA for migraine prophylaxis could also involve the inhibition of the release of CGRP from sensitized craniofacial muscles.

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References

[1] Alam Z, Coombes N, Waring RH, Williams AC, Steventon GB. Plasma levels of neuroexcitatory amino acids in patients with migraine or tension headache. J Neurol Sci 1998;156:102–6.
[2] Amano N, Hu JW, Sessle BJ. Responses of neurons in feline trigeminal subnucleus caudalis (medullary dorsal horn) to cutaneous, intraoral, and muscle afferent stimuli. J Neurophysiol 1986;55:227–43.
[3] Ambalavanar R, Moritani M, Moutanni A, Gangula P, Yallampalli C, Dessem D. Deep tissue inflammation upregulates neuropeptides and evokes nociceptive behaviors which are modulated by a neuropeptide antagonist. Pain 2006;120:53–68.
[4] Aoki KR. Review of a proposed mechanism for the antinociceptive action of botulinum toxin type A. Neurotoxicology 2005;26:785–93.
[5] Ashkenazi A, Silberstein S. Botulinum toxin type A for the treatment of headache: why we say yes. Arch Neurol 2008;65:146–9.
[6] Ashkenazi A, Silberstein S. Is botulinum toxin useful in treating headache? Yes. Curr Treat Options Neurol 2009;11:18–23.
[7] Baad-Hansen L, Cairns BE, Ernberg M, Svensson P. Effect of systemic monosodium glutamate (MSG) on headache and pericranial muscle sensitivity. Cephalalgia 2009;30:68–76.
[8] Burnett CA, Fartash L, Murray B, Lamey PJ. Masseter and temporalis muscle EMG levels and bite force in migraineurs. Headache 2000;40:813–7.

[9] Burstein R, Cutrer MF, Yarnitsky D. The development of cutaneous allodynia during a migraine attack clinical evidence for the sequential recruitment of spinal and supraspinal nociceptive neurons in migraine. Brain 2000;123:1703–9.
[10] Burstein R, Dodick D, Silberstein S. Migraine prophylaxis with botulinum toxin A is associated with perception of headache. Toxicon 2009;54:624–7.
[11] Burstein R, Yarnitsky D, Goor-Aryeh I, Ransil BJ, Bajwa ZH. An association between migraine and cutaneous allodynia. Ann Neurol 2000;47:614–24.
[12] Cady RK. OnabotulinumtoxinA (botulinum toxin type-A) in the prevention of migraine. Expert Opin Biol Ther 2010;10:289–98.
[13] Cairns BE, Dong X, Mann MK, Svensson P, Sessle BJ, Arendt-Nielsen L, McErlane KM. Systemic administration of monosodium glutamate elevates intramuscular glutamate levels and sensitizes rat masseter muscle afferent fibers. Pain 2007;132:33–41.
[14] Cairns BE, Gambarota G, Dunning PS, Mulkern RV, Berde CB. Activation of peripheral excitatory amino acid receptors decreases the duration of local anesthesia. Anesthesiology 2003;98:521–9.
[15] Cairns BE, Gambarota G, Svensson P, Arendt-Nielsen L, Berde CB. Glutamate-induced sensitization of rat masseter muscle fibers. Neuroscience 2002;109:389–99.
[16] Cairns BE, Sessle BJ, Hu JW. Characteristics of glutamate-evoked temporomandibular joint afferent activity in the rat. J Neurophysiol 2001;85:2446–54.
[17] Cairns BE, Svensson P, Wang K, Hupfeld S, Graven-Nielsen T, Sessle BJ, Berde CB, Arendt-Nielsen L. 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;90:2098–105.
[18] Caputi CA. Effectiveness of BoNT-A in the treatment of migraine and its ability to repress CGRP release. Headache 2004;44:837–8.
[19] Carlton SM, McNearney TA, Cairns BE. Peripheral glutamate receptors: Novel targets for analgesics? Seattle: IASP Press; 2003.
[20] Chun YH, Frank D, Lee JS, Zhang Y, Auh QS, Ro JY. Peripheral AMPA receptors contribute to muscle nociception and c-fos activation. Neurosci Res 2008;62:97–104.
[21] Cui M, Khanijou S, Rubino J, Aoki KR. Subcutaneous administration of botulinum toxin A reduces formalin-induced pain. Pain 2004;107:125–33.
[22] Dodick D, Blumenfeld A, Silberstein SD. Botulinum neurotoxin for the treatment of migraine and other primary headache disorders. Clin Dermatol 2004;22:76–81.
[23] Dong XD, Mann MK, Sessle BJ, Arendt-Nielsen L, Svensson P, Cairns BE. Sensitivity of rat temporalis muscle afferent fibers to peripheral N-methyl-D-aspartate receptor activation. Neuroscience 2006;141:939–45.
[24] Durham PL, Cady R. Regulation of calcitonin gene-related peptide secretion from trigeminal nerve cells by botulinum toxin type A: implications for migraine therapy. Headache 2004;44:35–42. discussion 42–33.
[25] Farinelli I, Coloprisco G, De Filippis S, Martelletti P. Long-term benefits of botulinum toxin type A (BOTOX) in chronic daily headache: a five-year long experience. J Headache Pain 2006;7:407–12.
[26] Farinelli I, De Filippis S, Coloprisco G, Missori S, Martelletti P. Future drugs for migraine. Intern Emerg Med 2009;4:367–73.
[27] Fernandez-de-las-Penas C, Cuadrado ML, Arendt-Nielsen L, Simons DG, Pareja JA. Myofascial trigger points and sensitization: an updated pain model for tension-type headache. Cephalalgia 2007;27:383–93.
[28] Ferrari A, Spaccapelo L, Pinetti D, Tacchi R, Bertolini A. Effective prophylactic treatments of migraine lower plasma glutamate levels. Cephalalgia 2009;29:423–9.
[29] Ferrari MD, Odink J, Bos KD, Malessy MJ, Bruyn GW. Neuroexcitatory plasma amino acids are elevated in migraine. Neurology 1990;40:1582–6.
[30] 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.
[31] Gazerani P, Pedersen NS, Staahl C, Drewes AM, Arendt-Nielsen L. Subcutaneous Botulinum toxin type A reduces capsaicin-induced trigeminal pain and vasomotor reactions in human skin. Pain 2009;141:60–9.
[32] Gazerani P, Staahl C, Drewes AM, Arendt-Nielsen L. The effects of Botulinum Toxin type A on capsaicin-evoked pain, flare, and secondary hyperalgesia in an experimental human model of trigeminal sensitization. Pain 2006;122:315–25.
[33] Goadsby PJ. Pathophysiology of migraine. Neurol Clin 2009;27:335–60.
[34] Goadsby PJ, Charbit AR, Andreou AP, Akerman S, Holland PR. Neurobiology of migraine. Neuroscience 2009;161:327–41.
[35] Guyuron B, Varghai A, Michelow BJ, Thomas T, Davis J. Corrugator supercilii muscle resection and migraine headaches. Plast Reconstr Surg 2000;106:429–34. discussion 435-427.
[36] Humeau Y, Doussau F, Grant NJ, Poulain B. How botulinum and tetanus neurotoxins block neurotransmitter release. Biochimie 2000;82:427–46.
[37] Jackson DL, Hargreaves KM. Activation of excitatory amino acid receptors in bovine dental pulp evokes the release of iCGRP. J Dent Res 1999;78:54–60.
[38] Jensen R. Pathogenic importance of muscular disorders in tension-type headache. Funct Neurol 1994;9:175–82.
[39] Jensen R, Fuglsang-Frederiksen A, Olesen J. Quantitative surface EMG of pericranial muscles in headache. A population study. Electroencephalogr Clin Neurophysiol 1994;93:335–44.
[40] Juhasz G, Zsombok T, Modos EA, Olajos S, Jakab B, Nemeth J, Szolcsanyi J, Vitrai J, Bagdy G. NO-induced migraine attack: strong increase in plasma calcitonin gene-related peptide (CGRP) concentration and negative correlation with platelet serotonin release. Pain 2003;106:461–70.
[41] Kim CC, Bogart MM, Wee SA, Burstein R, Arndt KA, Dover JS. Predicting migraine responsiveness to botulinum toxin type A injections. Arch Dermatol 2010;146:159–63.
[42] Lam DK, Sessle BJ, Hu JW. Glutamate and capsaicin effects on trigeminal nociception II: activation and central sensitization in brainstem neurons with deep craniofacial afferent input. Brain Res 2009;1253:48–59.
[43] Lassen LH, Haderslev PA, Jacobsen VB, Iversen HK, Sperling B, Olesen J. CGRP may play a causative role in migraine. Cephalalgia 2002;22:54–61.
[44] Lee JS, Ro JY. Peripheral metabotropic glutamate receptor 5 mediates mechanical hypersensitivity in craniofacial muscle via protein kinase C dependent mechanisms. Neuroscience 2007;146:375–83.
[45] Luvisetto S, Marinelli S, Cobianchi S, Pavone F. Anti-allodynic efficacy of botulinum neurotoxin A in a model of neuropathic pain. Neuroscience 2007;145:1–4.
[46] Mann MK, Dong XD, Svensson P, Cairns BE. Influence of intramuscular nerve growth factor injection on the response properties of rat masseter muscle afferent fibers. J Orofac Pain 2006;20:325–36.
[47] McMahon HT, Foran P, Dolly JO, Verhage M, Wiegant VM, Nicholls DG. Tetanus toxin and botulinum toxins type A and B inhibit glutamate, gamma-aminobutyric acid, aspartate, and met-enkephalin release from synaptosomes. Clues to the locus of action. J Biol Chem 1992;267:21338–43.
[48] Morch CD, Hu JW, Arendt-Nielsen L, Sessle BJ. Convergence of cutaneous, musculoskeletal, dural and visceral afferents onto nociceptive neurons in the first cervical dorsal horn. Eur J Neurosci 2007;26:142–54.
[49] Moskowitz MA. Basic mechanisms in vascular headache. Neurol Clin 1990;8:801–15.
[50] Obermann M, Diener HC. Is botulinum toxin useful in treating headache? No. Curr Treat Options Neurol 2009;11:24–31.
[51] Omote K, Kawamata T, Kawamata M, Namiki A. Formalin-induced release of excitatory amino acids in the skin of the rat hindpaw. Brain Res 1998;787:161–4.
[52] Park HJ, Lee Y, Lee J, Park C, Moon DE. The effects of botulinum toxin A on mechanical and cold allodynia in a rat model of neuropathic pain. Can J Anaesth 2006;53:470–7.
[53] Pearce LB, First ER, MacCallum RD, Gupta A. Pharmacologic characterization of botulinum toxin for basic science and medicine. Toxicon 1997;35:1373–412.
[54] Pedersen-Bjergaard U, Nielsen LB, Jensen K, Edvinsson L, Jansen I, Olesen J. Calcitonin gene-related peptide, neurokinin A and substance P: effects on nociception and neurogenic inflammation in human skin and temporal muscle. Peptides 1991;12:333–7.
[55] Pikoff H. Is the muscular model of headache still viable? A review of conflicting data. Headache 1984;24:186–98.
[56] Purkiss JR, Welch MJ, Doward S, Foster KA. Capsaicin stimulates release of substance P from dorsal root ganglion neurons via two distinct mechanisms. Biochem Soc Trans 1997;25:542S.
[57] Ro JY, Zhang Y, Nies M. Substance P does not play a critical role in neurogenic inflammation in the rat masseter muscle. Brain Res 2005;1047:38–44.
[58] Schulte-Mattler WJ, Leinisch E. Evidence based medicine on the use of botulinum toxin for headache disorders. J Neural Transm 2008;115:647–51.
[59] Simpson LL. The origin, structure, and pharmacological activity of botulinum toxin. Pharmacol Rev 1981;33:155–88.
[60] Sprenger T, Goadsby PJ. Migraine pathogenesis and state of pharmacological treatment options. BMC Med 2009;7:71.
[61] Stovner LJ, Zwart JA, Hagen K, Terwindt GM, Pascual J. Epidemiology of headache in Europe. Eur J Neurol 2006;13:333–45.
[62] Tvedskov JF, Lipka K, Ashina M, Iversen HK, Schifter S, Olesen J. No increase of calcitonin gene-related peptide in jugular blood during migraine. Ann Neurol 2005;58:561–8.
[63] Villalon CM, Olesen J. The role of CGRP in the pathophysiology of migraine and efficacy of CGRP receptor antagonists as acute antimigraine drugs. Pharmacol Ther 2009;124:309–23.
[64] Welch MJ, Purkiss JR, Foster KA. Sensitivity of embryonic rat dorsal root ganglia neurons to Clostridium botulinum neurotoxins. Toxicon 2000;38:245–58.