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Subcutaneous Botulinum toxin type A reduces capsaicin-induced trigeminal pain and vasomotor reactions in human skin

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ABSTRACT

The present human study aimed at investigating the effect of subcutaneous administration of Botulinum toxin type A (BoNT/A) on capsaicin-induced trigeminal pain, neurogenic inflammation and experimentally induced cutaneous pain modalities. Fourteen healthy males (26.3 ± 2.6 years) were included in this double-blind and placebo-controlled trial. The subjects received subcutaneous BoNT/A (22.5 U) and isotonic saline in the mirror sides of their forehead. Pain and neurogenic inflammation was induced by four intradermal injections of capsaicin (100 μg/μL) (before, and days 1, 3 and 7 after treatments). The capsaicin-induced pain intensity, pain area, the area of secondary hyperalgesia, the area of visible flare and vasomotor reactions were recorded together with cutaneous heat, electrical and pressure pain thresholds. BoNT/A reduced the capsaicin-induced trigeminal pain intensity compared to saline ($F = 37.9$, $P < 0.001$). The perceived pain area was smaller for the BoNT/A-treated side compared to saline ($F = 7.8$, $P < 0.05$). BoNT/A reduced the capsaicin-induced secondary hyperalgesia ($F = 5.3$, $P < 0.05$) and flare area ($F = 10.3$, $P < 0.01$) compared to saline. BoNT/A reduced blood flow ($F_{1,26} = 109.5$, $P < 0.001$) and skin temperature ($F_{1,26} = 63.1$, $P < 0.001$) at the capsaicin injection sites compared to saline and its suppressive effect was maximal at days 3 and 7 ($P < 0.05$, post hoc test). BoNT/A elevated cutaneous heat pain thresholds ($F = 17.1$, $P < 0.001$) compared to saline; however, no alteration was recorded for electrical or pressure pain thresholds ($P > 0.05$). Findings from the present study suggest that BoNT/A appears to preferentially target Cfibers and probably TRPV1-receptors, block neurotransmitter release and subsequently reduce pain, neurogenic inflammation and cutaneous heat pain threshold.

1. Introduction

Botulinum toxin type A (BoNT/A) is one of the seven serotypes (A–G) of botulinum neurotoxins derived from *Clostridium botulinum* [38]. BoNT/A inhibits the release of acetylcholine at neuromuscular junctions, which causes a flaccid paralysis of affected muscles. Its pharmacological action is through the cleavage of SNAP-25 (synaptosome-associated protein of 25 kDa) in motor nerve terminals [23,41]. BoNT/A also affects other cholinergic synapses in salivary [34] and sweat glands [46] and it is used for glandular hyperactivity.

BoNT/A has been beneficial in number of diseases or conditions with unwanted muscle hyperactivity, e.g. spasticity, dystonia and drooling [25,39,55]. Patients treated with BoNT/A for such conditions also noted a remarkable reduction of pain [12,55]. Since then, the analgesic effects of BoNT/A have been investigated in multiple forms of pain, e.g. headache disorders. A recent review [47] indicated an overall negative results from randomized, controlled trials on the effect of BoNT/A for the most common primary headaches. In those studies, the prospectively defined primary endpoints were not met, but secondary outcomes were positive in some of the studies [19,35,45,47,50,51].

Research into the effects of BoNT/A in headache field is ongoing, which may be due to the idea that BoNT/A may still be considered as an option for subgroups of patients. To predict who will benefit from the injections is yet unknown.

To explore the mechanism of potential analgesic action of the BoNT/A, several studies have been conduced. *In vitro* studies have shown the inhibitory effect of BoNT/A on proinflammatory neuropeptides release [36,43,58]. Aanimal investigations have also demonstrated antinociceptive action of BoNT/A in both inflammatory and neuropathic pain models [7,16,33,40]. Several experimental studies have examined the analgesic effects of BoNT/A in healthy volunteers with discrepant results. Blersch et al. [10] and Voller

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et al. [57] found no effect of BoNT/A on electrical and heat pain thresholds in human skin. The study by Krämer et al. [30] also failed to detect any antinociceptive or antihyperalgesic effect of BoNT/A in an electrically induced pain model. However, a reduction of the neurogenic flare was observed. In an Ultraviolet B pain model no analgesic or anti-inflammatory effect of BoNT/A was seen [53]. In contrast to the above-mentioned studies, we showed that intramuscular BoNT/A inhibits the capsaicin-evoked pain and neurogenic vasodilatation in human skin mostly due to toxin leakage to the skin in the forehead [21]. Tugnoli et al. [56] showed pain and flare reduction in the forearm skin; however, such result could not be repeated by Schulte-Mattler et al. [48] in a similar model.

To further investigate the mechanism(s) underlying the action of BoNT/A, the present study was designed to clarify the modality specific analgesia and blocking profile on different nerve fiber populations in human skin. The time course of the effects following the application of BoNT/A were also addressed up to 1 week after the injection.

The novelty of this study is to investigate if subcutaneous BoNT/A has specific analgesic action on particular cutaneous pain modalities and also to explore how fast the subcutaneous BoNT/A acts in the human skin.

2. Methods

2.1. Subjects and design

Volunteers were recruited through local announcements at Aalborg University, Denmark. Sixteen healthy right-handed males were screened and fourteen (23–32 yr, mean ± SD 26.3 ± 2.6 years) were included. A lack of personal interest and a state of illness were recorded as reasons for the two screen-failures. Initial screening involved recording of demographic information, review of medical history and a physical examination. Subjects were excluded if they had previous or present systemic, skin or neuromuscular diseases, psychiatric disorders or a history of severe allergy. Volunteers were asked to refrain from the use of any medication, and restrict the use of alcohol and caffeine. Participants were fully informed about the goal, procedure and safety aspects of the study and written informed consent was obtained from all of them prior to the start of the study.

The study was designed as a randomized, double-blind and placebo-controlled trial and was approved by the regional Ethics Committee (Counties of Nordjylland and Viborg, Denmark; VN-20060026), the Danish Medicines Agency (2612-3160) and the Danish Data Protection Agency. The study was carried out in accordance with the Good Clinical Practice (GCP) guidelines and the Declaration of Helsinki. Any adverse events were recorded by the investigator according to the guidelines of GCP.

The study was performed at the research laboratories of Aalborg Hospital and Aalborg University, Denmark.

Each subject participated in five visits after screening/recruitment as shown schematically in Fig. 1A. Subjects were randomized to receive placebo (saline) or BoNT/A in left or right side of their foreheads at the research laboratory at Aalborg Hospital, which is equipped with all facilities in case of emergencies or severe reactions.

It is known that the effect of botulinum injections on nearby skin wrinkles makes it difficult to mount a truly blinded study. However, we designed the study in such a way that the study nurse who did the randomization and prepared the syringes did not take part in the injection and measurement procedures. The injection

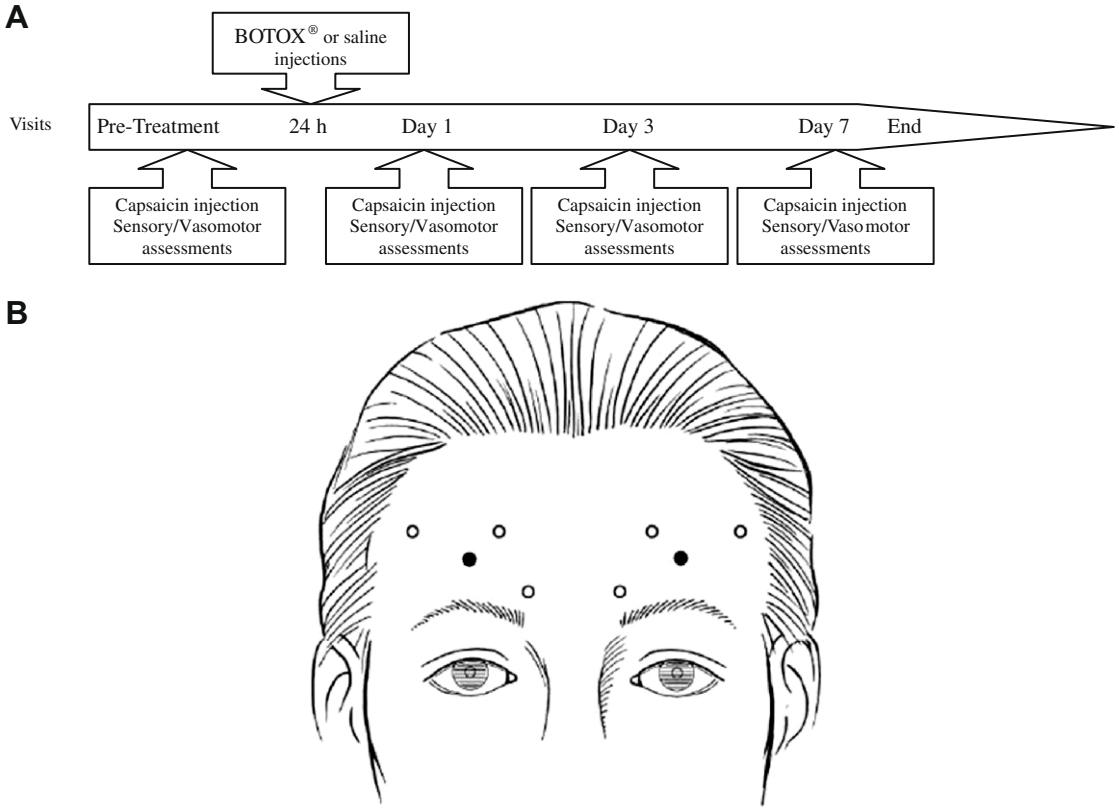

Fig. 1. (A) Simplified experimental design representing visits and injections. (B) Schematic injection sites for BoNT/A and saline. Filled circles show capsaicin injection sites and unfilled circles represent BoNT/A or saline injection sites (two injections above the frontalis muscle and one injection above the corrugator muscle at either side for each treatment).

syringes were prepared in such a way that the physician (NP) performing the injections could not recognize whether saline or BoNT/A was injected. Subjects and investigator (PG) were also blinded for the BoNT/A/saline side during the whole study. Additionally, after treatments, the subjects were asked to guess the BoNT/A-injected side. Possible answers were "right", "left" or "unknown".

2.2. BoNT/A and saline injections

Each vial of BoNT/A (BOTOX®, Allergan Inc., Irvin, CA; 100 U/vial) was reconstituted with non-preserved saline solution (0.9%), as recommended by the manufacturer. BoNT/A (total dose 22.5 U) was then injected in the forehead area subcutaneously above the frontalis (two sites, each 7.5 U) and corrugator (one site, 7.5 U) muscles (Fig. 1B). The dose used in this study is in the range of units used for headache treatments (i.e. 20–100 U, [6]). The same volume of sterile physiological saline (0.9%) was injected in the mirror forehead as control.

Electromyographic (EMG)-guided injections (Clavis™, Medtronic, Copenhagen, Denmark) with disposable hypodermic needle electrodes (Bo-ject™27G, Medtronic, Skovlunde, Denmark) were used. This method [27] ensured that the injections were made in subcutaneous and not into the muscles to avoid muscular effect of BoNT/A. It has been reported that BoNT/A diffuses within 1.5–2 cm radius [11] and small doses of BoNT/A minimizes the spread of the toxin to the underlying muscles [52]. Thus, we expected a small local area of toxin spreading in the skin after its subcutaneous injections.

Subjects were rested in a supine position on a bed during the injections. Prior to all injections the skin was washed by soap and water and dried. Alcohol was not used to prevent toxin deactivation.

2.3. Capsaicin pain model

The capsaicin pain model and related measurements were performed in a quiet laboratory at Aalborg University (mean temperature ± SD: 21.0 ± 1.0 °C) equipped with Epipen® 0.3 mg (Meridian Medical Techn. Inc. St. Louis, USA), in case of anaphylactic reaction.

Capsaicin (100 μg/0.1 ml, vehicle consisted of 1% Tween 80, 1% ethanol and 98% physiological saline, Aalborg Hospital Pharmacy, Aalborg, Denmark) was injected intradermally into the skin above the frontalis muscle (Fig. 1B) at each visit, except the BoNT/A/saline injection visit (Fig. 1A). Single use tuberculin syringes (1 ml), fitted with 27-gauge disposable needles were used. Prior to capsaicin injection, the skin was cleaned with alcohol and was allowed to be completely dried before the needle insertion.

2.4. Assessments

2.4.1. Pain intensity and duration

Each volunteer rated the capsaicin-evoked pain sensation continuously using an electronic visual analogue scale (VAS) of 0 (no pain) to 10 (worst pain imaginable). The pain intensity was rated until the subjects indicated that they no longer felt any pain. The data were sampled every second and recorded on the computer's hard disk for off-line analysis. The maximum pain intensity and the duration of pain were calculated.

2.4.2. Pain area

Subjects were asked to outline their pain area on a standard pattern of the human face. The distribution pattern was outlined at the maximum pain sensation. The area was calculated later (ACECAD, model D9000 + digitizer, Taiwan).

2.4.3. Flare

The area of flare reaction (the reddening of the skin around the injection site) was measured by tracing the border of flare onto transparent papers 5 min after the capsaicin injection. The area was calculated later (ACECAD, model D9000 + digitizer, Taiwan).

2.4.4. Blood flow

The superficial blood flow was measured at the sites of injections using a laser Doppler imaging system (Moor Instruments, Devon, UK). The device produces an output signal proportional to the blood cell perfusion (or flux). The laser head was positioned 30 cm above the measurement site. The scan region was 7.5 × 7.5 cm. Measurements of blood flow were taken for 5 min prior to capsaicin injection and 5–10–20 min post injections. The mean blood flow was calculated using relative flux (arbitrary units).

2.4.5. Skin temperature

Measurements of skin temperature were taken by means of a thermography camera (ThermoVision A40-M, Flir systems AB, Danderyd, Sweden). The pictures from whole face were taken for 5 min prior to capsaicin injection and 5–10–20 min post injections. The temperature resolution of the device was 0.08 °C. Thermographic images were stored on computer's hard disk for off-line analysis of the profile and local changes of skin temperature.

2.4.6. The area of secondary hyperalgesia

The area of secondary hyperalgesia was assessed when the capsaicin-induced pain was vanished. A handheld von Frey nylon monofilament (Somedic's Aesthesiometer No. 17, bending force 60.0 g, Somedic Sales AB, Hörby, Sweden) was used [15]. Volunteers were asked to keep their eyes closed. Stimulation was started approximately 6 cm away from the site of injection and was repeated along a pattern of eight radial linear paths. With movement along each line at steps of 1 cm with an interval of 2 s, the volunteers were asked to report the sensation of the pricking changed to a "different sensation", "unpleasant" or "burning pain". The points were then marked and traced on transparent sheets and the area was calculated.

2.4.7. Cutaneous heat pain threshold

Heat pain threshold was assessed by the method of limits [59]. A thermal sensory analyzer (TSA 2001, Medoc™, Ramat Yishai, Israel) was used for application of heat. The computer controlled Peltier thermode (size: 30 × 30 mm) was placed at the study site of the forehead. The baseline temperature was set at 32.0 °C. The rate of temperature change was +1 °C/s and the temperature range was 32–50 °C. The subjects were asked to press a button and stop the increase of temperature at the first perception of unpleasant heat. Each test was repeated two times and the mean was calculated for further statistical analysis. There was a 2 min inter-stimulus interval between each test.

2.4.8. Cutaneous electrical pain threshold

The stimulation was applied via a constant current stimulator (Stimulus Isolator model A 365, World Precision Instruments, Inc, Sarasota, Florida, USA). Disposable surface skin electrodes (Ambu Neuroline 720 electrodes, Ambu A/S, Ballerup, Denmark) were placed at the study site of the forehead to determine the electrical pain threshold. The intensity of the electrical stimuli (in mA) was raised (1 mA/s) until the subject reported the first sensation of the current (sensation threshold). Then the current was increased until the sensation changed to pricking-like pain (electrical pain threshold).

Measurements were recorded two times in a 2 min interval and the mean values were used for further statistical analysis.

2.4.9. Pressure pain threshold (PPT)

PPT measurements were performed with a hand-held algometer (Somedic algometer, Somedic Sales AB, Hörby, Sweden) mounted with a 1-cm diameter circular rubber probe calibrated

in kPa. To assess the PPT, the probe was held perpendicularly at the frontalis muscles at the capsaicin injection site, and pressure increased at a constant rate of 30 kPa/s. The PPT value where the subject felt the first change from pressure sensation to pain was recorded. When PPT was reached, the volunteers were instructed to press a button that froze the actual pressure on a digital display. The mean of three measurements on each point with an interval of 2 min between the measurements was considered the PPT. The PPT was determined at pre-injection and again 15 min after the capsaicin injection. The percentage of change was used for statistical analysis.

3. Statistical analysis

All values are presented as mean and standard errors of the mean (SE) in the text and figures.

Since each participant was exposed to both treatments and the measurements performed at four time points; data were analyzed with two-way repeated measures ANOVA (RM ANOVA) for two factors of treatment (BoNT/A *versus* Saline) and trials (repeated factor: pre-treatment, days 1, 3 and 7). Holm-Sidak Test was used as *post hoc* test.

When the Kolmogorov–Smirnov normality test failed, the data were transformed logarithmically. To avoid the loss of zero values in case of transformation, a constant of 0.1 was added to all raw data (zero and non-zero values, [9]).

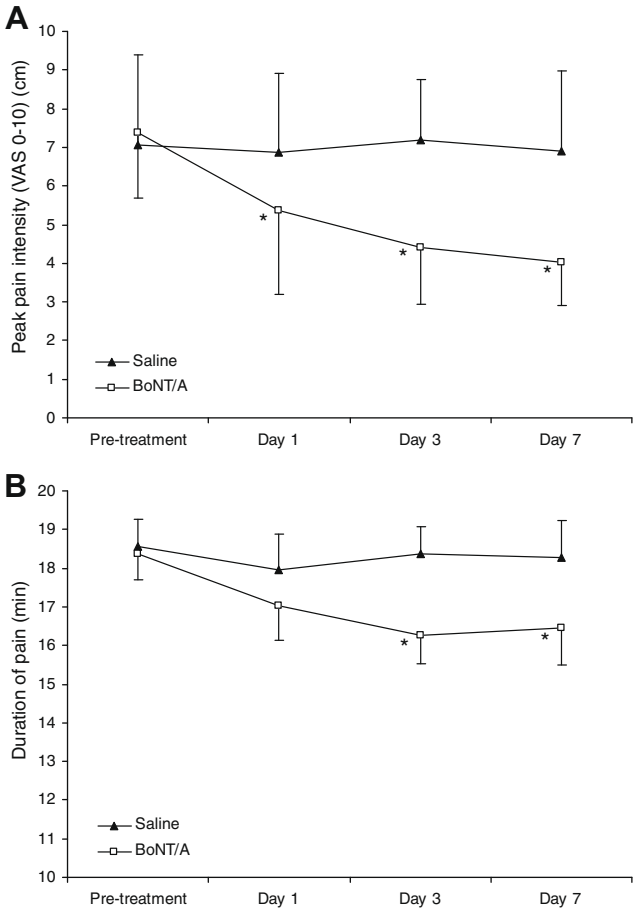

Fig. 2. (A) Peak pain intensity (visual analogue scale (VAS) score in 0–10 cm) and (B) duration of pain (min) following capsaicin-induced trigeminal pain in BoNT/A- and saline-treated forehead. * Indicates that BoNT/A induced a significantly lower pain intensity ($P < 0.001$) and shorter duration of pain ($P < 0.01$) compared to saline.

Sigmastat version 3.0 (SPSS Inc., Chicago, US) was used for statistical evaluation and $P < 0.05$ was considered as significant.

4. Results

All subjects completed the study. No side effect, e.g. weakness in the muscles or skin alteration following BoNT/A injections was reported. Only one subject reported general tiredness for the first days after BoNT/A injection that was resolved before the last visit at day 7 without taking any action. This was not categorized as an adverse event specific to BoNT/A according to the guidelines of GCP.

Nine (64.3%) participants chose "unknown" and three (21.4%) guessed the wrong side for the BoNT/A-injected side. Two participants (14.3%) could find the correct BoNT/A-injected side at day 7. Thus, the blinding procedure was successful.

4.1. Pain intensity and duration

BoNT/A reduced the capsaicin-induced trigeminal pain intensity compared to saline ($F_{1,39} = 37.9$, $P < 0.001$). The effect was comparable to saline at all time points after the treatments ($P < 0.05$, *post hoc* test) (Fig. 2A). The duration of capsaicin-induced trigeminal pain was shorter for the BoNT/A-treated side compared to saline ($F_{1,39} = 9.9$, $P < 0.01$) at days 3 and 7 ($P < 0.05$, *post hoc* test) (Fig. 2B).

4.2. Pain area

Volunteers drew smaller areas of capsaicin-induced trigeminal pain for BoNT/A- treated side compared to saline ($F_{1,39} = 7.8$, $P < 0.05$) at days 3 and 7 ($P < 0.05$, *post hoc* test).

The effect of the treatments (BoNT/A and saline) and trials on capsaicin-induced trigeminal pain area (cm^2) together with superimposed drawings on face-charts are shown in Fig. 3. BoNT/A and saline were given randomly to either side of the foreheads. To better illustrate the distribution pattern of the pain, all BoNT/A-injected sides are superimposed in one side, regardless of the actual side of the injection. Care was taken to correctly transfer the direction of the pain spreading, where it was mirrored. The same procedure was performed for saline.

4.3. The area of secondary hyperalgesia

The capsaicin-induced secondary hyperalgesia was reduced by BoNT/A treatment compared to saline ($F_{1,39} = 5.3$, $P < 0.05$) at day 7 ($P < 0.05$, *post hoc* test).

The effect of the treatments (BoNT/A and saline) and trials on capsaicin-induced secondary hyperalgesia is illustrated by Fig. 4.

4.4. Flare area

BoNT/A reduced the capsaicin-induced flare area compared to saline ($F_{1,39} = 10.3$, $P < 0.01$) at days 1 and 3 ($P < 0.05$, *post hoc* test). Fig. 5 illustrates the influence of treatment on capsaicin-induced flare area (cm^2) together with superimposed drawings on face-charts. BoNT/A and saline were given randomly to either side of the foreheads. To better illustrate the distribution pattern of the flare, all BoNT/A-injected sides are superimposed in one side, regardless of the actual side of the injection. Care was taken to correctly transfer the direction of the redness spreading, where it was mirrored. The same procedure was performed for saline.

4.5. Blood flow

BoNT/A reduced the capsaicin-induced elevated blood flow compared to saline ($F_{1,26} = 109.5$, $P < 0.001$) at days 3 and 7 ($P < 0.05$, *post hoc* test). After BoNT/A treatment the blood flow

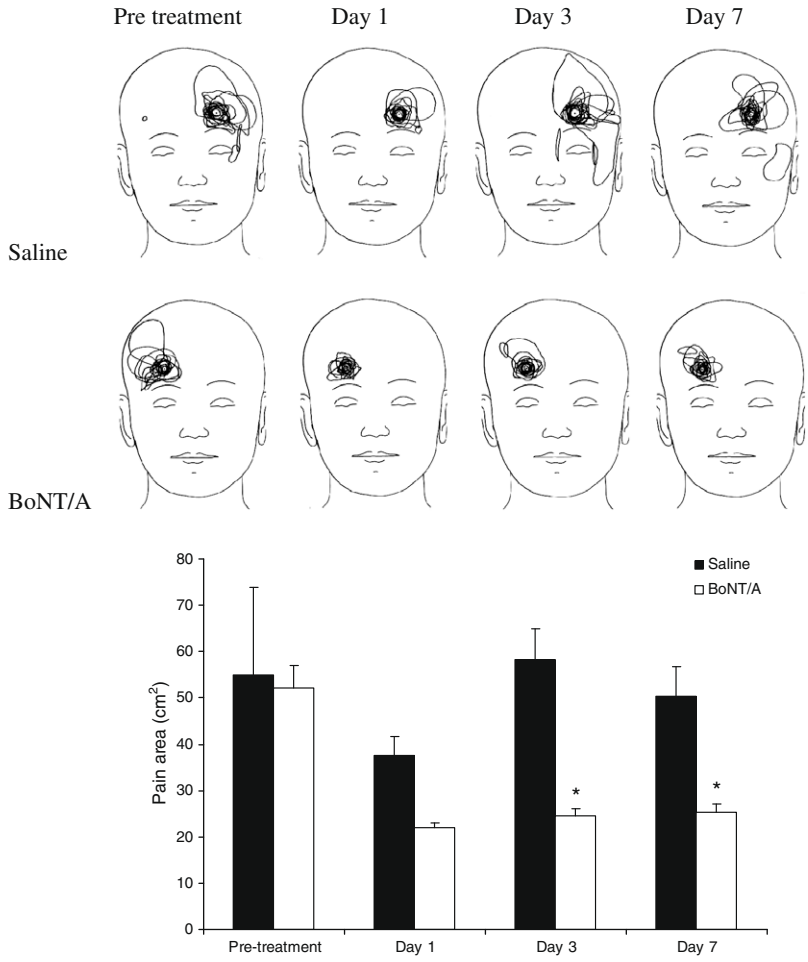

Fig. 3. Pain area (cm^2) following capsaicin-induced trigeminal pain in BoNT/A- and saline-treated foreheads. Superimposed face-charts of the capsaicin-induced trigeminal pain areas at maximum pain sensation are shown for saline (top) and BoNT/A-treated sides (down). * Indicates that BoNT/A significantly reduced capsaicin-induced trigeminal pain areas compared to saline ($P < 0.05$).BoNT/A and saline were given randomly to the either side of the foreheads. To better illustrate the distribution pattern of the pain, all BoNT/A-injected sides are superimposed in one side, regardless of the actual side of the injection. The same procedure was performed for saline.

change at days 3 and 7 was greater than day 1 ($P < 0.05$, *post hoc* test).

Typical laser Doppler images following the capsaicin challenge is shown in Fig. 6A. The effect of the treatments on blood flow is illustrated by Fig. 6B.

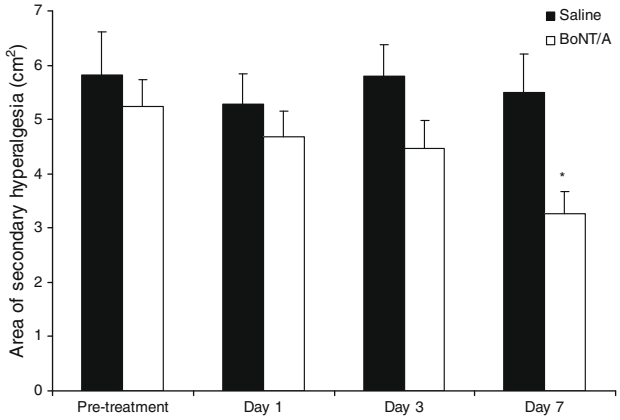

Fig. 4. The area of capsaicin-induced secondary hyperalgesia (cm^2) in BoNT/A and saline-treated foreheads. * Indicates that BoNT/A significantly reduced capsaicin-induced secondary hyperalgesic area compared to saline ($P < 0.05$).

4.6. Skin temperature

BoNT/A reduced the capsaicin-induced elevated skin temperature compared to saline ($F_{1,26} = 63.1$, $P < 0.001$) at days 3 and 7 ($P < 0.05$, *post hoc* test). After BoNT/A treatment the temperature change at days 3 and 7 was greater than day 1 ($P < 0.05$, *post hoc* test).

Typical thermographic images of the face are shown in Fig. 7A. The effect of the treatments on local temperature is depicted in Fig. 7B.

4.7. Cutaneous heat pain threshold

BoNT/A treatment increased heat pain threshold compared to saline ($F_{1,39} = 17.1$, $P < 0.001$) at days 3 and 7 ($P < 0.05$, *post hoc* test). After BoNT/A treatment the heat pain threshold was higher at day 7 compared with day 1 ($P < 0.05$, *post hoc* test).

The effects of treatments and trials on heat pain threshold are summarized in Table 1.

4.8. Cutaneous electrical pain threshold

BoNT/A treatment did not change cutaneous electrical pain threshold compared to saline ($F_{1,39} = 0.297$, $P = 0.595$).

The effects of treatments and trials on cutaneous electrical pain threshold are presented in Table 1.

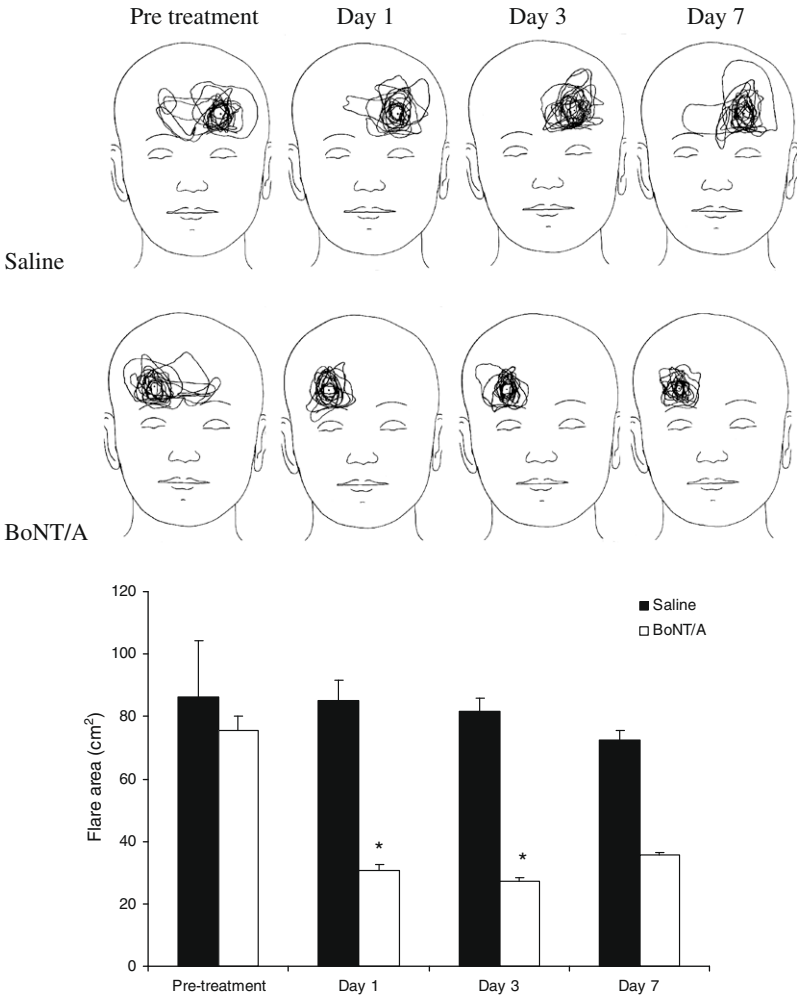

Fig. 5. Flare area (cm^2) following capsaicin-induced trigeminal pain in BoNT/A- and saline-treated foreheads. Superimposed face-charts of the capsaicin-induced flare areas are shown for saline (top) and BoNT/A-treated sides (down). * Indicates that BoNT/A significantly reduced capsaicin-induced flare areas compared to saline ($P < 0.01$).BoNT/A and saline were given randomly to the either side of the foreheads. To better illustrate the distribution pattern of the flare, all BoNT/A-injected sides are superimposed in one side, regardless of the actual side of the injection. The same procedure was performed for saline.

4.9. Pressure pain threshold

No significant alteration was seen in PPT due to BoNT/A treatment ($F_{1,39} = 1.2$, $P = 0.294$ as compared to saline). A summary of the effects of treatments and trials on PPT are given in Table 1.

5. Discussion

The present study demonstrated that subcutaneous BoNT/A reduced capsaicin-induced trigeminal pain, sensitization and neurogenic inflammation. BoNT/A altered cutaneous heat pain threshold, but had no effect on electrical or pressure pain thresholds. The earliest analgesic effect of BoNT/A was recorded at 24 h after its application.

5.1. The effect of subcutaneous BoNT/A on pain

The mechanisms of a direct analgesic effect of BoNT/A are not fully clarified yet. The analgesic effect of BoNT/A; however, seems to be distinct from BoNT/A effect on muscles. This idea is supported by the evidences showing the effect of BoNT/A on pain appears in no muscle contraction or lasts for longer duration of time after relieving of muscle contraction [2].

One possibility is that BoNT/A influences on neurons involved in pain perception and prevents the release of other neurotransmitters than acetylcholine [4,18]. There are evidences to support such hypothesis. Sufficient *in vitro* exposure to BoNT/A has been shown to reduce the release of glutamate, substance P, CGRP and vasopressin from cultured cells or isolated materials [17,24,36,42]. The point is that *in vitro* studies require very large doses, long exposure period of time or using some techniques to enhance the internalization of the toxin. However, seeing the same effect *in vivo* on pain transmission requires normal dose and exposure.

Pre-clinical studies were also successful to demonstrate that BoNT/A is able to inhibit pain and neuropeptide release, e.g. in rat formalin test [16]. Antinociceptive effects of BoNT/A was also seen in a rat capsaicin pain model, which supports our present findings. Pre-treatment of the rat foot by BoNT/A reduced the frequency of foot withdrawal in response to pressure and elevated temperature caused by capsaicin injection [7].

Human experimental models of pain have provided more information about the analgesic effects of BoNT/A. However, despite of *in vivo* and *in vitro* studies, there is a result discrepancy in human experimental studies [10,21,30,48,53,56,57]. Such diversity suggest that the analgesic efficacy of BoNT/A may depend on the injection site, route of administration, dose and outcome measures as well as timing of the challenge, applied pain model and also the BoNT/A preparation itself.

We used intradermal capsaicin pain model in human skin. This substance by a unique molecular mechanism, the stimulation of

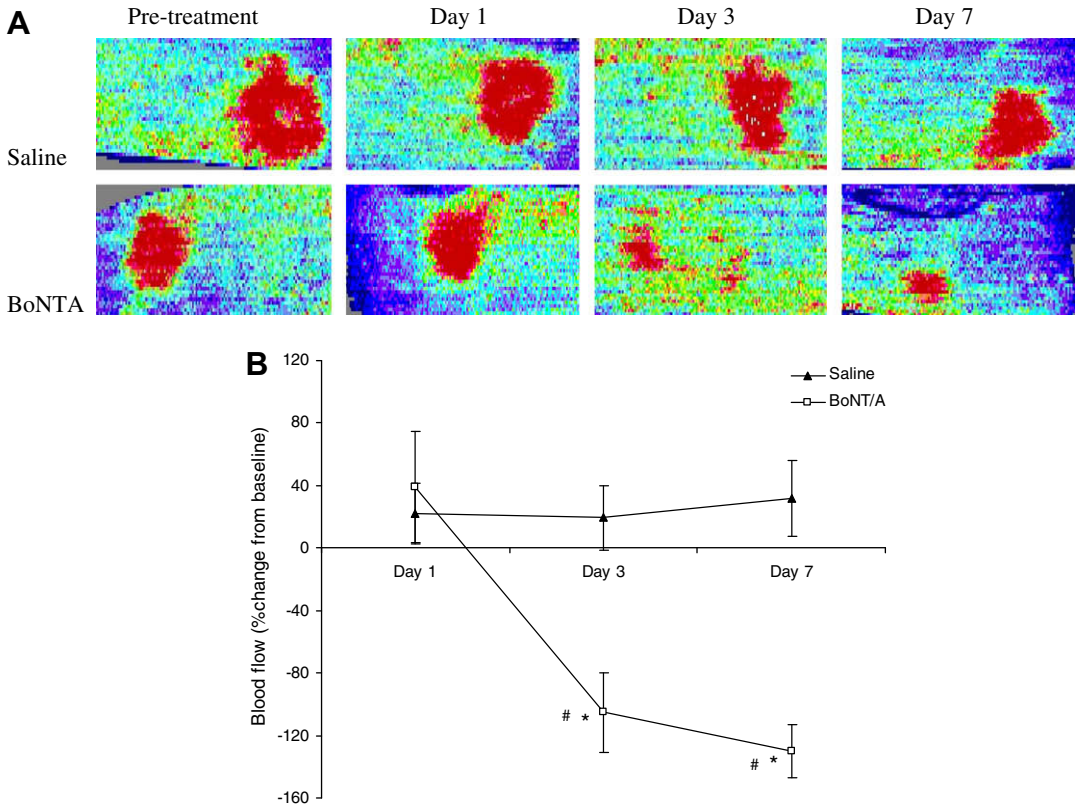

Fig. 6. (A) Typical laser Doppler images 5 min after capsaicin-induced pain in saline (top) and BoNT/A-treated sides (down) from the same subject. (B) Blood flow change (%) following capsaicin-induced pain in BoNT/A- and saline-treated sides. * Indicates significant difference between BoNT/A- and saline-treated sides ($P < 0.001$). # indicates the greater effect of BoNT/A at days 3 and 7 ($P < 0.05$) as compared to day 1.

the transient receptor potential vanilloid receptor 1 (TRPV1-receptors) [14], causes pain and neurogenic inflammation [31,49].

Our results clearly showed that BoNT/A modulates the pain induced by intradermal injection of capsaicin, in agreement with Tugnoli et al. [56] findings in the forearm skin. On the basis of the above-mentioned evidences from *in vitro* and *in vivo* studies, it is proposed that BoNT/A interacts with molecules involved in pain perception, e.g. substance P, CGRP and glutamate. Such neurotransmitters are released by primary sensory terminals and probably the ability of BoNT/A to block their release, may cause the reduction of pain and/or sensitization [18].

5.2. The effect of subcutaneous BoNT/A on neurogenic inflammation

We recorded a clear reduction of capsaicin-induced flare area, capsaicin-induced elevated skin blood flow and local skin temperature in BoNT/A-treated skin compared with saline. A reduction in blood flow in the capsaicin treated footpad was also seen following pretreatment with BoNT/A [Francis et al., Personal Communication, Toxins 2005, Denver, USA]. Krämer et al. [30] demonstrated reduced electrically induced flare by BoNT-A, while Voller et al. [57] and Sycha et al. [53] found no effect.

Neurogenic inflammation reflects the release of neuropeptides (e.g. CGRP and substance P) from peripheral nociceptive nerve endings that leads to dilatation of peripheral blood vessels manifesting as redness and heat. BoNT/A may also alter the release of various agents that also affect blood flow [4].

The lack of complete blockade of flare by capsaicin as it is seen in the present study may be due to a partial rather than complete C-fiber inhibition and consequently neuropeptide release. A portion of BoNT/A molecules may bind to C-fibers after the injection; whether a significant number of BoNT/A molecules are needed to inhibit these fibers to reach the complete inhibition effect is not clear yet [2]. Another possibility is that there may be BoNT/A – sensitive and BoNT/A – non-sensitive C-fibers. The number of activated fibers releasing neuropeptides may also be critical, which differ in the healthy volunteers compared to the pathological and clinical conditions [2].

5.3. The effect of subcutaneous BoNT/A on the area of secondary hyperalgesia

Capsaicin causes an altered sensation by sensitization of central neurons, a phenomenon defined as secondary hyperalgesia [32]. The effect of BoNT/A on central sensitization was seen in rat models of neuropthic pain [8,33,40]. The anti-allodynic effect of BoNT/A was seen from the day after the injection and maintained at least for 3 weeks [33]. In the present study, BoNT/A reduced the area of secondary hyperalgesia at day 7 after its application. Our previous study, using intramuscular BoNT/A in a similar pain model, also showed such an effect [21]. However, in other human experimental pain models, Voller et al. [57] and Krämer et al. [30] did not find any difference in hyperalgesic area treated by BoNT/A or placebo.

In the present study, the reduction in the area of secondary hyperalgesia could be a consequence of the capsaicin-evoked pain reduction [28] or probably due to an indirect central effect of BoNT/A [1,2]. If BoNT/A blocks the transmitter release, peripheral sensitization of nociceptors would be reduced as a consequence. Then the nociceptive signals into the central nervous system and manifestations of central sensitization would be indirectly reduced [18].

5.4. The effect of subcutaneous BoNT/A on heat pain threshold

In the present study, BoNT/A significantly increased the heat pain threshold, which was lowered by intradermal capsaicin

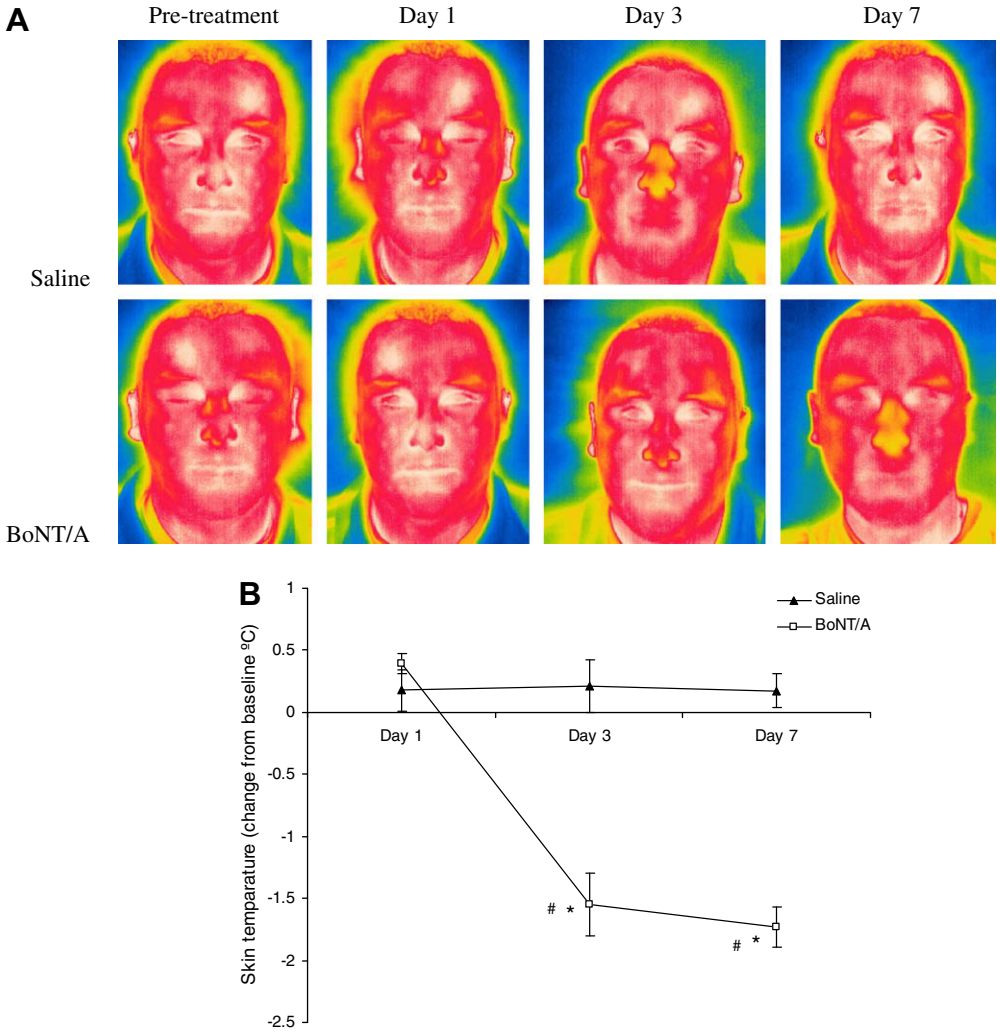

Fig. 7. (A) Typical thermographic images 5 min after capsaicin-induced pain in saline (top) and BoNT/A-treated sides (down) from the same subject. (B) Temperature change (°C) following capsaicin-induced pain in BoNT/A- and saline-treated sides. * Indicates significant difference between BoNT/A- and saline-treated sides ($P < 0.001$). # indicates the greater effect of BoNT/A at days 3 and 7 ($P < 0.05$) as compared to day 1.

injection. Our result is in contrast with Blersch et al. [10], Voller et al. [57] and Tugnoli et al. [56] studies that did not show any alteration in heat pain threshold. One possible explanation for the effect of BoNT/A on heat pain threshold is the effect of toxin on TRPV1-receptors located on sensory nerve endings, which is known as a target for capsaicin [14]. TRPV1-receptors are expressed on the plasma membrane via SNAREs (Soluble *N*-ethylmaleimide-sensitive factor attachment receptors)-dependent exocytosis, which is inhibited by BoNT/A [37]. This phenomenon is supported by data from clinical investigations on patients with intractable detrusor overactivity. In these patients the levels of TRPV1 in biopsy samples were elevated, which was reduced by BoNT/A treatment [3]. Studies with TRPV1 knockout mice showed that genetic removal of TRPV1 did not affect responses to acute noxious thermal stimuli, but impaired the animal ability to develop thermal hyperalgesia [13]. Thus, at least in part, the influence of BoNT/A on heat pain threshold, could be because of its effect on peripheral TRPV1-receptors, as essential receptors for inflammatory thermal hyperalgesia.

5.5. The effect of subcutaneous BoNT/A on electrical pain threshold

The electrical pain threshold after the capsaicin injection was decreased, but not altered by BoNT/A compared with saline. This result is in agreement with the studies done by Blersch et al. [10], Voller et al. [57] and Schulte-Mattler et al. [48]. In preclinical

Table 1
Cutaneous heat (°C), electrical (mA) and pressure pain (kPa) thresholds following capsaicin-induced pain in BoNT/A- and saline-treated sides.

	Pre-treatment		Day 1		Day 3		Day 7	
	BoNT/A	Saline	BoNT/A	Saline	BoNT/A	Saline	BoNT/A	Saline
Heat pain threshold (°C)	38.2 ± 2.5	38.2 ± 1.9	39.8 ± 1.9	38.4 ± 2.4	41.0 ± 1.5*	38.2 ± 2.1	42.9 ± 3.8*	38.5 ± 2.4
Electrical pain threshold (mA)	58.2 ± 3.7	58.6 ± 5.9	60.3 ± 4.9	63.9 ± 4.6	65.4 ± 4.0	58.9 ± 4.7	64.3 ± 4.4	62.1 ± 4.2
Pressure pain threshold (kPa)	226.6 ± 65.7	255.7 ± 77.8	224.9 ± 65.6	238.7 ± 47.4	226.2 ± 57.8	222.3 ± 45.1	227.6 ± 95.1	233.2 ± 55.6

* Indicates significant difference between BoNT/A- and saline-treated sides ($P < 0.001$).

studies, BoNT/A does not directly affect the nociceptive nerve transmission of acute pain [2]. The unchanged electrical thresholds in BoNT/A-treated areas, suggest that BoNT/A may not influence the perception of acute pain caused by direct fiber activation.

5.6. The effect of subcutaneous BoNT/A on pressure pain threshold

It has been shown that the cutaneous component accounts for approximately 45–70% of the pressure detection threshold [26,29] and localized application of the pressure can activate the cutaneous nociceptors because of the shear force around the probe [22]. On the other hand, application of topical local anaesthetics to the facial skin has revealed minor or no changes in the PPTs, suggesting a modest role of superficial inputs to the PPTs [20,44,54]. In the present study, PPT remained unchanged at the BoNT/A- and saline-treated areas. In a pilot study in patients with postherpetic neuralgia treated by subcutaneous botulinum injections, however, the PPT was higher at the BoNT/A-treated area. Interestingly, those patients with trigeminal presentation were better responders than the other regions such as thoracic and lumbar [5]. Again, existence of fiber differences between the healthy compared to the pathological and clinical conditions [2] may explain the effect seen in those patients.

In conclusion, the observed analgesia may be caused by a local peripheral effect of BoNT/A on nociceptive fibers. BoNT/A appears to preferentially target C-fibers and probably TRPV1-receptors, block neurotransmitter/neuropeptide release and subsequently reduce pain, neurogenic inflammation and heat pain thresholds. Further experiments, using pharmacological combinations of BoNT/A with e.g., glutaminergic or TRPV1 agonist/antagonists as well as neurochemical markers may provide better understanding of the interaction of BoNT/A with the neurotransmitter system in pain modulation.

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References

[1] Aoki KR. Evidence for antinociceptive activity of botulinum toxin type A in pain management. Headache 2003;43:S9–S15.
[2] Aoki KR. Review of a proposed mechanism for the antinociceptive action of botulinum toxin type A. Neurotoxicology 2005;26:785–93.
[3] Apostolidis A, Popat R, Yiangou Y, Cockayne D, Ford AP, Davis JB, et al. Decreased sensory receptors P2X3 and TRPV1 in suburothelial nerve fibers following intradetrusor injections of botulinum toxin for human detrusor overactivity. J Urol 2005;174:977–82.
[4] Arezzo JC. Possible mechanisms for the effects of botulinum toxin on pain. Clin J Pain 2002;18:S125–32.
[5] Argoff CE. A focused review on the use of botulinum toxins for neuropathic pain. Clin J Pain 2002;18:S177–81.
[6] Ateş Y. Botulinum toxin for the treatment of headaches: a review of current practices and evidence based-data. Agri 2006;18:5–11.
[7] Bach-Rojecky L, Lacković Z. Antinociceptive effect of botulinum toxin type a in rat model of carrageenan and capsaicin induced pain. Croat Med J 2005;46:201–8.
[8] Bach-Rojecky L, Relja M, Lackovic Z. Botulinum toxine type A in experimental neuropathic pain. J Neural Transm 2005;112:215–9.
[9] Bartlett MS. The use of transformations. Biometrics 1947;3:39–52.
[10] Blersch W, Schulte-Mattler WJ, Przywara S, May A, Bigalke H, Wohlfarth K. Botulinum toxin A and the cutaneous nociception in humans: a prospective, double-blind, placebo-controlled, randomized study. J Neurol Sci 2002;205:59–63.
[11] Braune C, Erbguth F, Birklein F. Dose thresholds and duration of the local anhidrotic effect of botulinum toxin injections: measured by sudometry. Br J Dermatol 2001;144:111–7.
[12] Brin MF, Fahn S, Moskowitz C, Friedman A, Shale HM, Greene PE, et al. Localized injections of botulinum toxin for the treatment of focal dystonia and hemifacial spasm. Mov Disord 1987;2:237–54.
[13] Caterina MJ, Leffler A, Malmberg AB, Martin WJ, Trafton J, Petersen-Zeitz KR, et al. Impaired nociception and pain sensation in mice lacking the capsaicin receptor. Science 2000;288:306–13.
[14] Caterina MJ. Transient receptor potential ion channels as participants in thermosensation and thermoregulation. Am J Physiol Regul Integr Comp Physiol 2007;292:R64–76.
[15] Cruccu G, Anand P, Attal N, Garcia-Larrea L, Haanpää M, Jørum E, et al. EFNS guidelines on neuropathic pain assessment. Eur J Neurol 2004;11:153–62.
[16] Cui M, Khanijou S, Rubino J, Aoki KR. Subcutaneous administration of botulinum toxin A reduces formalin-induced pain. Pain 2004;107:125–33.
[17] Cui M, Li S, You S, Khanijou S, Aoki KR. Mechanisms of the antinociceptive effect of subcutaneous Botox inhibition of peripheral and central nociceptive processing (abstract). Naunyn Schmiedebergs Arch Pharmacol 2002;365:R17.
[18] Dolly JO, Aoki KR. The structure and mode of action of different botulinum toxins. Eur J Neurol 2006;13:1–9.
[19] Evers S, Vollmer-Haase J, Schwaag S, Rahmann A, Husstedt IW, Frese A. Botulinum toxin A in the prophylactic treatment of migraine–a randomized, double-blind, placebo-controlled study. Cephalalgia 2004;24:443–838.
[20] Fujisawa M, Shoji S, Ishibashi K, Clark GT. Pressure pain threshold with and without iontophoretic anesthesia of the masseter muscle in asymptomatic males. J Orofac Pain 1999;13:97–103.
[21] 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.
[22] Greenspan JD, McGillis SL. Stimulus features relevant to the perception of sharpness and mechanically evoked cutaneous pain. Somatosens Mot Res 1991;8:137–47.
[23] Humeau Y, Doussau F, Grant NJ, Poulain B. How botulinum and tetanus neurotoxins block neurotransmitter release. Biochimie 2000;82:427–46.
[24] Ishikawa H, Mitsui Y, Yoshitomi T, Mashimo K, Aoki S, Mukuno K, et al. Presynaptic effects of botulinum toxin type A on the neuronally evoked response of albino and pigmented rabbit iris sphincter and dilator muscles. Jpn J Ophthalmol 2000;44:106–9.
[25] Jankovic J, Schwartz KS. Use of botulinum toxin in the treatment of hand dystonia. J Hand Surg [Am] 1993;18:883–7.
[26] Jensen K, Andersen HO, Olesen J, Lindblom U. Pressure pain threshold in human temporal region. Evaluation of a new pressure algometer. Pain 1986;25:313–23.
[27] Klein AW, Mantell A. Electromyographic guidance in injecting botulinum toxin. Dermatol Surg 1998;24:1184–6.
[28] Koltzenburg M, Lundberg LE, Torebjork HE. Dynamic and static components of mechanical hyperalgesia in human hairy skin. Pain 1992;51:207–19.
[29] Kosek E, Ekholm J, Hansson P. Pressure pain thresholds in different tissues in one body region. The influence of skin sensitivity in pressure algometry. Scand J Rehabil Med 1999;31:89–93.
[30] Krämer HH, Angerer C, Erbguth F, Schmelz M, Birklein F. Botulinum Toxin A reduces neurogenic flare but has almost no effect on pain and hyperalgesia in human skin. J Neurol 2003;250:188–93.
[31] LaMotte RH, Lundberg LE, Torebjork HE. Pain, hyperalgesia and activity in nociceptive C units in humans after intradermal injection of capsaicin. J Physiol 1992;448:749–64.
[32] LaMotte RH, Shain CN, Simone DA, Tsai EF. Neurogenic hyperalgesia psychophysical studies of underlying mechanisms. J Neurophysiol 1991;66:190–211.
[33] 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.
[34] Mancini F, Zangaglia R, Cristina S, Sommaruga MG, Martignoni E, Nappi GC. Double-blind, placebo-controlled study to evaluate the efficacy and safety of botulinum toxin type A in the treatment of drooling in parkinsonism. Mov Disord 2003;18:685–8.
[35] Mathew NT, Frishberg BM, Gawel M, Dimitrova R, Gibson J, Turkel C. Botulinum toxin type A (BOTOX) for the prophylactic treatment of chronic daily headache: a randomized, double-blind, placebo-controlled trial. Headache 2005;45:293–307.
[36] 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.
[37] Morenilla-Palao C, Planells-Cases R, García-Sanz N, Ferrer-Montiel A. Regulated exocytosis contributes to protein kinase C potentiation of vanilloid receptor activity. J Biol Chem 2004;279:25665–72.
[38] Oguma K, Fujinaga Y, Inoue K. Structure and function of *Clostridium botulinum* toxins. Microbiol Immunol 1995;39:161–8.
[39] Pacchetti C. Double-blind, placebo-controlled study to evaluate the efficacy and safety of botulinum toxin type A in the treatment of drooling in parkinsonism. Mov Disord 2003;18:685–8.

[40] 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.
[41] Pearce LB, First ER, MacCallum RD, Gupta A. Pharmacologic characterization of botulinum toxin for basic science and medicine. Toxicon 1997;35:1373–412.
[42] Purkiss J, Welch M, Doward S, Foster K. Capsaicin-stimulated release of substance P from cultured dorsal root ganglion neurons: involvement of two distinct mechanisms. Biochem Pharmacol 2000;59:1403–6.
[43] 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.
[44] Reid KI, Carlson C, Rayens MK, Gracely RH. The influence of cutaneous tissue afferents on masticatory pain-pressure thresholds. J Orofac Pain 1996; 10:324–9.
[45] Saper JR, Mathew NT, Loder EW, DeGryse R, VanDenburgh AM. A double-blind randomised placebo-controlled exploratory study comparing injection sites with low doses of botulinum toxin type A in the prevention of episodic migraine. J Neurol 2005;252. II-58.
[46] Schnider P, Binder M, Auff E, Kittler H, Berger T, Wolff K. Double-blind trial of botulinum A toxin for the treatment of focal hyperhidrosis of the palms. Br J Dermatol 1997;136:548–52.
[47] Schulte-Mattler WJ, Leinisch E. Evidence based medicine on the use of botulinum toxin for headache disorders. J Neural Transm 2008;115:647–51.
[48] Schulte-Mattler WJ, Opatz O, Blersch W, May A, Bigalke H, Wohlfahrt K. Botulinum toxin A does not alter capsaicin-induced pain perception in human skin. J Neurol Sci 2007;260:38–42.
[49] Serra J, Campero M, Ochoa J. Flare and hyperalgesia after intradermal capsaicin injection in human skin. J Neurophysiol 1998;80:2801–10.
[50] Silberstein SD, Göbel H, Jensen R, Elkind AH, DeGryse R, Walcott JM, et al. Botulinum toxin type A in the prophylactic treatment of chronic tension-type headache: a multicentre, double-blind, randomized, placebo-controlled, parallel-group study. Cephalalgia 2006;26:790–800.
[51] Silberstein SD, Stark SR, Lucas SM, Christie SN, Degryse RE, Turkel CC. Botulinum toxin type A for the prophylactic treatment of chronic daily headache: a randomized, double-blind, placebo-controlled trial. Mayo Clin Proc 2005;80:1126–37.
[52] Swartling C, Färnstrand C, Abt G, Stålberg E, Naver H. Side-effects of intradermal injections of botulinum A toxin in the treatment of palmar hyperhidrosis: a neurophysiological study. Eur J Neurol 2001;8:451–6.
[53] Sycha T, Samal D, Chizh B, Lehr S, Gustorff B, Schnider P, et al. A lack of antinociceptive or antiinflammatory effect of botulinum toxin A in an inflammatory human pain model. Anesth Analg 2006;102: 509–16.
[54] Takahashi K, Taguchi T, Itoh K, Okada K, Kawakita K, Mizumura K. Influence of surface anesthesia on the pressure pain threshold measured with different-sized probes. Somatosens Mot Res 2005;22:299–305.
[55] Tsui JK, Eisen A, Stoessl AJ, Calne S, Calne DB. Double-blind study of botulinum toxin in spasmodic torticollis. Lancet 1986;2:245–7.
[56] Tugnoli V, Capone JG, Eleopra R, Quatrale R, Sensi M, Gastaldo E, et al. Botulinum toxin type A reduces capsaicin-evoked pain and neurogenic vasodilatation in human skin. Pain 2007;130:76–83.
[57] Voller B, Sycha T, Gustorff B, Schmetterer L, Lehr S, Eichler HG, et al. A randomized, double-blind, placebo-controlled study on analgesic effects of botulinum toxin A. Neurology 2003;61:940–4.
[58] Welch MJ, Purkiss JR, Foster KA. Sensitivity of embryonic rat dorsal root ganglia neurons to *Clostridium botulinum* neurotoxins. Toxicon 2000;38:245–58.
[59] Yarnitsky D, Sprecher E, Zaslansky R, Hemli JA. Heat pain thresholds: normative data and repeatability. Pain 1995;60:329–32.