Journal of Oral Rehabilitation

Journal of Oral Rehabilitation 2012 **39**; 63–72

Review Article

Intradermal injection of Botulinum toxin type A alleviates infraorbital nerve constriction-induced thermal hyperalgesia in an operant assay

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SUMMARY **Recent studies have shown that infraorbital nerve constriction (IoNC)-induced mechanical allodynia has been attenuated by administration of highly purified 150-kDa Botulinum neurotoxin type A (BoNT/A). Here, we extend these studies to determine whether BoNT/A could attenuate IoNC-induced symptoms of thermal hyperalgesia. Instead of testing head withdrawal thresholds, a thermal operant assay was used to evaluate cortical processing of sensory input following IoNC. In this assay, a fasted rat's desire to obtain a food reward (sweetened condensed milk) is coupled to its ability to tolerate facial contact with a warm (45 °C) thermode. Bilateral IoNC decreased the ratio of thermode contact duration/event, which is an indicative of thermal hyperalgesia. BoNT/A injection intradermally in the area of infraorbital nerve (IoN) innervation 7 days after IoNC resulted in decreased number of facial contacts and increased the ratio of contact duration/event (measured at 14 days after IoNC). The BoNT/A (2–200 pg) effects were dose dependent and statistically significant at 100 and 200 pg ($P < 0{\cdot}05$). Complete reversal of thermal hyperalgesia symptoms was obtained with a 200-pg dose, without affecting sham rat behaviour. Off-site (neck) injection of BoNT/A did not relieve thermal hyperalgesia, while co-injection of BoNT/A with a neutralising antibody in the area of IoN innervation prevented relief of thermal hyperalgesia. Neither IoNC nor BoNT/A injection affected operant assay parameters with a 24 °C thermode, indicating selectivity of thermal hyperalgesia measurements. These results strongly suggest that intradermal injection of BoNT/A in the area of IoN innervation alleviates IoNC-induced thermal hyperalgesia in an operant assay.**

KEYWORDS: **Botulinum toxin, thermal hyperalgesia, trigeminal ganglion, thermal stimulation**

Accepted for publication 3 June 2011

Introduction

Orofacial neuropathic pain patients often present with symptoms of (in decreasing order of prevalence): spontaneous pain, abnormal mechanical sensitivity (most often dynamic allodynia), cold allodynia and hyperalgesia and heat hypersensitivity (1, 2). Various rodent models of peripheral nerve injury have been developed, which mimic human neuropathy symptoms of tactile allodynia, thermal hyperalgesia and spontaneous pain (3–7). Measuring these symptoms in animal neuropathy models designed to develop the much

doi: 10.1111/j.1365-2842.2011.02236.x

needed new therapies is a challenge, with nociceptive withdrawal thresholds from mechanical or thermal stimulus being the most common measures of symptomatology. Assessment of trigeminal nerve-mediated nociceptive responses has been limited to a handful of methods that assess processing within the brain stem (e.g. withdrawal responses or grooming) (7–10) elicited using Von Frey filaments (7) or thermal stimulation (11). Non-operant assessments of innate behaviours do not reveal cerebral processing of nociception. In this study, we utilised a thermal operant facial testing system to evaluate cortical input following infraorbital nerve constriction (IoNC) (12).

Our research goal is to develop new treatments that decrease chronic pain while minimising side effects. One strategy is to target peripheral sensory neurons whose excitability and neurotransmitter release is increased in chronic pain states (13). Such targeted treatments, which do not penetrate the blood–brain-barrier, should theoretically limit central side effects. One such treatment is Botulinum toxin (BoNT), which reportedly blocks vesicular neurotransmitter release by disabling the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex proteins which mediate vesicular transmitter release (14, 15). Indeed, clinicians have used BoNT, outside the product licence, to treat chronic pain symptoms not associated with muscle spasms (16). Previous studies demonstrated that BoNT is capable of attenuating release of substance P, calcitonin gene-related peptide (CGRP) or glutamate from sensory neurons in culture (17–20), in isolated preparations (21) and *in vivo* (22). We recently reported that IoNC-induced tactile allodynia and vesicular transmitter release from trigeminal ganglion (TRG) neurons were both reduced after peripheral injection of purified Botulinum neurotoxin type A (BoNT/A) in the area of IoN innervation (23). Here, we report that IoNC in rats produces thermal hyperalgesia in an operant assay and demonstrates that peripheral injection of BoNT/A (2–200 pg) dose dependently alleviates the thermal hyperalgesia of IoNC.

Materials and methods

Trigeminal neuropathy model

All procedures regarding animal usage in this study were performed in accordance with specifications of an animal protocol approved by Okayama University (OKU-2009091). Male Sprague Dawley rats (200–300 g) ($n = 28$) were anesthetised by intraperitoneal injection with ketamine (35 mg kg^{-1}) and xylazine (5 mg kg^{-1}), the infraorbital branch of the trigeminal nerve was exposed bilaterally and two silk ligatures (4–0) were loosely tied around the IoN at about 2 mm apart (7, 23). To obtain the desired degree of constriction, a criterion formulated by Bennett and Xie (3) was applied: The ligations reduced the diameter of the nerve by a just noticeable amount and retarded but did not occlude the circulation through the superficial vasculature. The skin incision was closed in layers using nylon sutures (4–0). Control rats were subjected to sham surgery, where the IoN was exposed using the above procedures, but not constricted.

Thermal operant testing

The rats were lightly anesthetised using sevoflurane (2·5%, inhalation), and their hair was bilaterally removed from the orofacial region using clippers, followed by depilatory cream 1 day prior to behavioural testing (12). Excess cream was removed with a moistened paper towel to minimise skin irritation. The rats were food and water fasted for 24 h prior to each testing session, and following each session provided with standard food chow and water *ad libitum*. The animals were brought into the behavioural procedure room 1 h prior to testing and allowed to acclimate to the temperature and ambient noise of the room.

Facial testing was completed using a reward-conflict operant testing paradigm as described previously (12, 24). Briefly, a testing cage (20·3 cm W × 20·3 cm D × 16·2 cm H) with acrylic walls was constructed with an opening in one wall (4 × 6 cm), which was lined with grounded metal (aluminium) tubing. The testing cage is custom made by one of the authors (JKN). The tubing served as a thermode when connected to a water pump (NCB-1200*) via flexible polyethylene tubing through which heated water (45 °C) or non-heated water (24 °C) as control was circulated. Stimulus thermode temperature was verified using a thermometer (Fluke 54†). A standard rodent watering bottle containing a diluted (1:2 with water) sweetened condensed milk solution‡ was mounted outside the

*Tokyo Rikakikai, Tokyo, Japan.
†Fluke, Everett, WA, USA.
‡Morinaga milk, Tokyo, Japan.

cage. The bottle was then positioned in proximity to the cage such that the animal could access to the bottle when simultaneously contacting the thermode with its face. The room temperature was maintained at 24 ± 1 °C for all behavioural tests. Unrestrained animals were placed separately in a testing cage, and the data acquisition system was activated (DI-148U/HS[§]). When the rat drank from the water bottle, the skin on its shaved face contacted the grounded thermode completing an electrical circuit (130 V). The closed circuit was registered in the computer, and data were collected at 60 Hz for the entire length of the experiment. The circuit was established from the metal thermode to the animal by grounding the floor with an aluminium sheet for recording of 'facial contact' events. The duration of each facial contact and the total number of events (facial contact) were recorded. The investigator monitored online data acquisition to ensure each recorded facial contact on the tubing.

Rats were trained twice a week for 2 weeks. Each 10-min training session was performed with the thermode at room temperature. Once trained, the facial testing region for each animal was depilated under light sevoflurane anaesthesia (2·5%, inhalation) once a week to maximise thermal stimulus contact. Baseline measurement began 2–3 days after the training and was performed three times every 3–4 days. Testing was repeated at 7 and 14 days post-surgery and data analysed offline.

During data analysis, the threshold for facial contact detection was set at 1·0 V, above background noise, to minimise false-positive event registration and events typically registered as >5·0 V. A facial contact event was registered when the signal went above threshold and ended when the signal dropped below threshold. The cumulative duration and frequency of events were determined for the facial stimulus contact data. The total amount of milk consumed (g) was measured and compared at each of the testing temperatures. Data were analysed using custom-written routines in LabView Express[¶] and Excel**.

Botulinum neurotoxin injection

After behavioural testing on post-operative day 7, the rats were anesthetised by i.p. injection with ketamine (35 mg kg^{-1}) and xylazine (5 mg kg^{-1}). The BoNT/A was purified by one of the authors (KO) (25, 26). Purified BoNT/A composed of a light and a heavy chain was administered as a single intradermal injection (0, 1, 50 or 100 pg in 0·1 mL of sterile saline) bilaterally [total dose of BoNT/A was 0, 2, 100 or 200 pg, 200-pg dose is equal to 20 units (20 MLD)] in the snout at the centre of the whisker pad (i.e. between rows B and C of the vibrissae) (n = 4 in each dose) or back of the neck of the anesthetised rats (n = 4) with Hamilton syringe (80330, 10 μL, 28 s/s''/s[††]). The bleb of the injection materials was absorbed within a day. In some rats, a mixture of BoNT/A 100 pg and polyclonal anti-BoNT/A antibody 150 ng was injected into the whisker pad (n = 4). The BoNT/A antibody was also generated by one of the authors (KO). It was generated by immunising rabbits with formalin-inactivated BoNT/A and then partially purified by ammonium sulphate precipitation followed by DEAE anion-exchange chromatography. The antibody is polyclonal, and the antibody specificity was determined by enzyme-linked immunosorbent assay (ELISA) and Western blotting methods (Fig. 1). The detail methods of ELISA and Western blotting were same as previous report (26). Thermal testing was repeated on post-operative day 14 (7 days after injection). The control group of sham rats was injected saline with intradermally.

Statistical analysis

Results are presented as group means ± s.e.m. Differences in group means of sham control without BoNT/A injection (Fig. 2) were evaluated by one-way RM ANOVA with Fisher *post hoc* test. Differences between baseline and IoNC (Fig. 3) and between IoNC and BoNT/A with the 24 °C thermode (Fig. 5) were evaluated by paired t-test. Also, differences between IoNC and BoNT/A injection (Fig. 4) were evaluated by two-way RM ANOVA with Fisher *post hoc* test. The Sigma Stat 3·11 software[‡‡] was used for these analyses.

The behaviour of sham surgery rats with the 45 °C thermode changed during the study period, and all data for IoNC rats were divided by the sham rat average data at the same time point. In this study, $P < 0.05$ was considered statistically significant.

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[¶]National Instruments Corporation, Austin, TX, USA.
**Microsoft, Redmond, WA, USA.
[††]Hamilton Co., Reno, NV, USA.
[‡‡]Systat, San Jose, CA, USA.

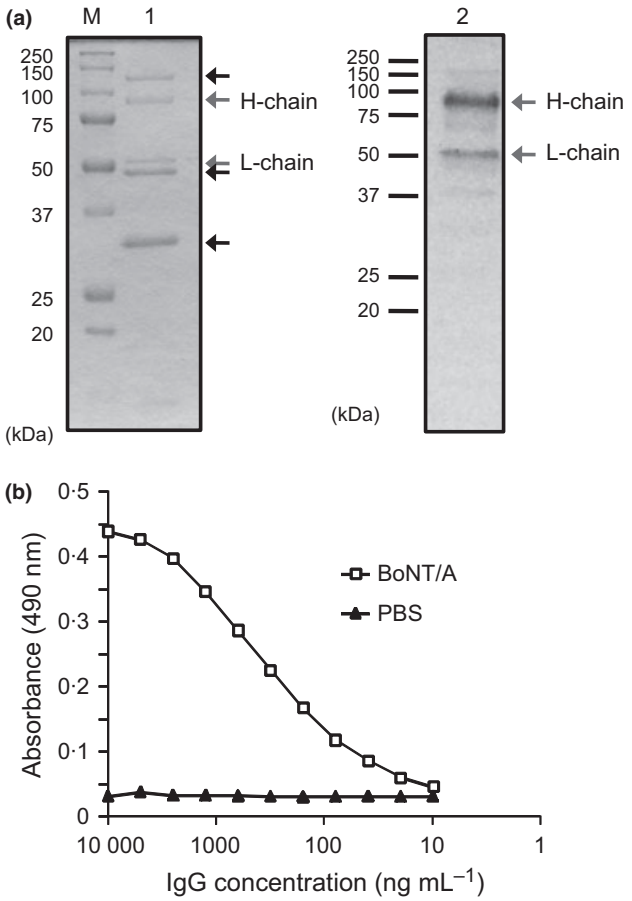

Fig. 1. Anti-Botulinum neurotoxin type A (BoNT/A) antibody specificity with Western blot and ELISA. (a) The progenitor toxin that is a complex of BoNT/A and non-toxic components was separated by SDS-PAGE and then subjected to Coomassie brilliant blue staining (lane 1). The Western blot analysis of the progenitor toxin and anti-BoNT/A antibody showed that the antibody specifically detected the heavy and light chains of BoNT/A (lane 2). Lane M is molecular size marker. (b) ELISA was performed using BoNT/A and anti-BoNT/A antibody serially diluted in twofold step. The ELISA data showed that the titration curve was dose dependent and anti-BoNT/A antibody binds to BoNT/A.

Results

Control rat behaviour in the operant assay

With each test session, sham surgery rats ($n = 4$) showed decreases in the number of contacts with the 45 °C thermode (Fig. 2a). Total contact time per session did not change during the study period (Fig. 2b), while the average duration of each contact increased (Fig. 2c). The behaviour of a separate group of sham surgery rats ($n = 4$) tested with the 24 °C thermode did not change (Fig 2d–f), suggesting that the behavioural changes were specific to the 45 °C thermode.

Effects of IoNC on operant outcome measures

Compared with baseline, measurements obtained at 7 days post-IoNC revealed a trend towards increased number of contact events (Fig. 3a) without changes in the total duration of contacts (Fig. 3b). However, the ratio of contact duration/contact number was significantly decreased after IoNC compared to the baseline data ($P < 0{\cdot}01$) (Fig. 3c). In previous studies with this thermal operant system, this ratio was shown to be a sensitive indicator of thermal hyperalgesia (12). Thus, IoNC rats increased the frequency of the 45 °C thermode contacts while decreasing the duration of each contact ($n = 16$). By contrast, no such changes were seen with another group of IoNC rats tested with the 24 °C thermode ($n = 4$) (Fig 3d–f), while the ratio of contact duration/contact number with the 24 °C thermode increased ($P = 0{\cdot}107$, sample size estimation $n = 10$) (Fig. 3f). The increase in the ratio of contact duration/contact number indicates no thermal hyperalgesia or no tactile allodynia with the 24 °C thermode. IoNC had no effect on total milk consumption in either the sham the IoNC 45 °C or the 24 °C thermode group (data not shown).

BoNT/A reverses the IoNC-induced changes in operant measures

BoNT/A administration reversed the effect of IoNC in the 45 °C thermal operant assay, as it decreased contact event numbers ($n = 4$ in each dose) ($P < 0{\cdot}05$ with 100 and 200 pg BoNT/A to the whisker pad injection) (Fig. 4a) without concomitant changes in the average total contact duration ($n = 4$ in each dose) (Fig. 4b). In effect, this increased the ratio of contact duration/contact numbers ($n = 4$ in each dose) ($P < 0{\cdot}01$ with 200 pg BoNT/A to the whisker pad injection) (Fig. 4c). Moreover, the BoNT/A effects were dose dependent, becoming statistically significant with the 200-pg doses. By contrast, BoNT/A injection had no discernable behavioural effects in the sham-operated rats ($n = 4$) (Fig. 4).

In a separate group of IoNC rats, intradermal BoNT/A injection at the back of the neck did not alleviate thermal hyperalgesia symptoms ($n = 4$) (Fig. 4),

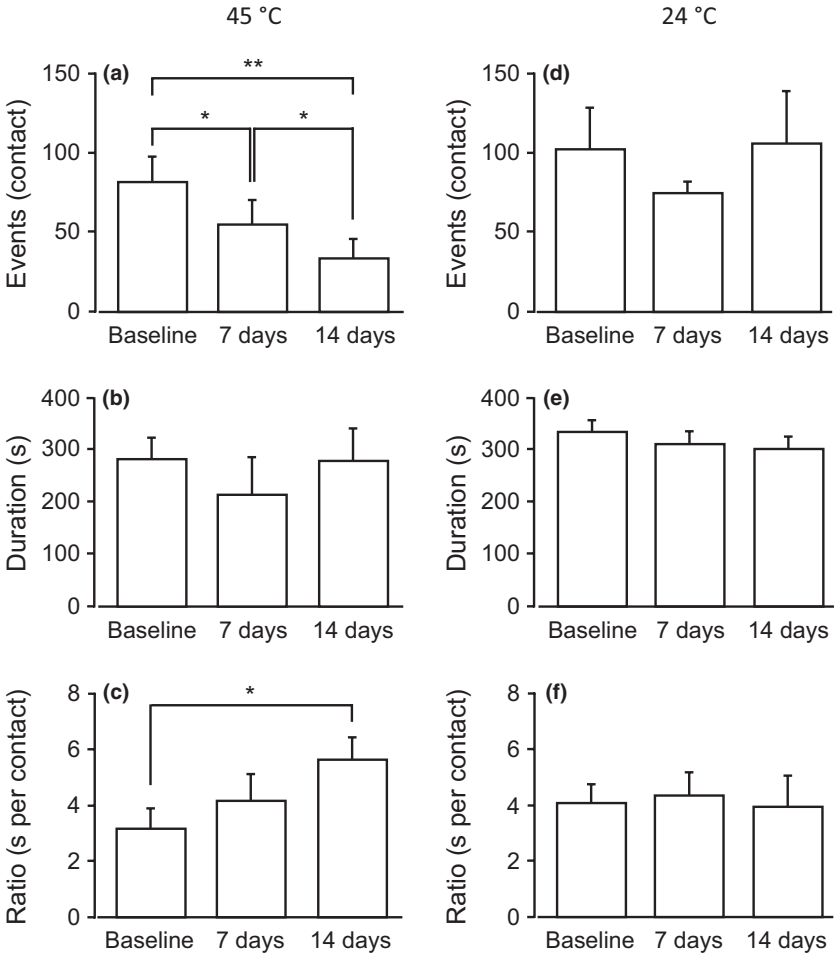

Fig. 2. Effects of sham surgery on operant outcome measures. In rats with the sham surgery ($n = 4$), the contact events at the 45 °C thermode appeared to decrease with each experimental session (a). The total contact time at each session did not change during the study period (b). The average contact duration (ratio of contact duration/contact number) appeared to increase with each experimental session (c). As the sham rat data were altered during the study period, subsequent data for IoNC rats were normalised to the sham rat data at the same time point. The sham surgery rats ($n = 4$) tested with the 24 °C thermode did not change (d–f), suggesting that the behavioural changes were specific to the 45 °C thermode. *$P < 0{\cdot}05$, **$P < 0{\cdot}01$, one-way RM ANOVA with Fisher *post hoc* test.

indicating specificity of BoNT/A actions for the innervation area of injured IoN neurons. Another group of IoNC rats was tested in the 45 °C thermal operant assay to address the selectivity of BoNT/A action. To do this, a mixture of BoNT/A and its neutralising antibody was injected bilaterally in the whisker pads ($n = 4$). This injection did not alleviate thermal hyperalgesia symptoms (Fig. 4), demonstrating selectivity of BoNT/A on decreasing the symptoms of thermal hyperalgesia.

Another set of IoNC rats that were previously tested with the 24 °C thermode (Fig. 3) was also treated with BoNT/A (200 pg) to reveal possible BoNT effects on the operant behaviours at the 24 °C thermode. BoNT/A injection at 7 days post-IoNC had no effect on operant behaviour at the 24 °C thermode ($n = 4$) (Fig. 5). These results provided further support for the specificity of BoNT/A effects on thermal hyperalgesia and the lack of tactile allodynia contribution to the rat operant behaviour when contact is made at the thermode.

Discussion

In this study, we demonstrated that IoNC produces thermal hyperalgesia in the region innervated by the IoN, which is consistent with the previous demonstrations that chronic constriction injury of the IoN produces behavioural alterations indicative of trigeminal neuropathic pain (7, 23, 27–29). Most of

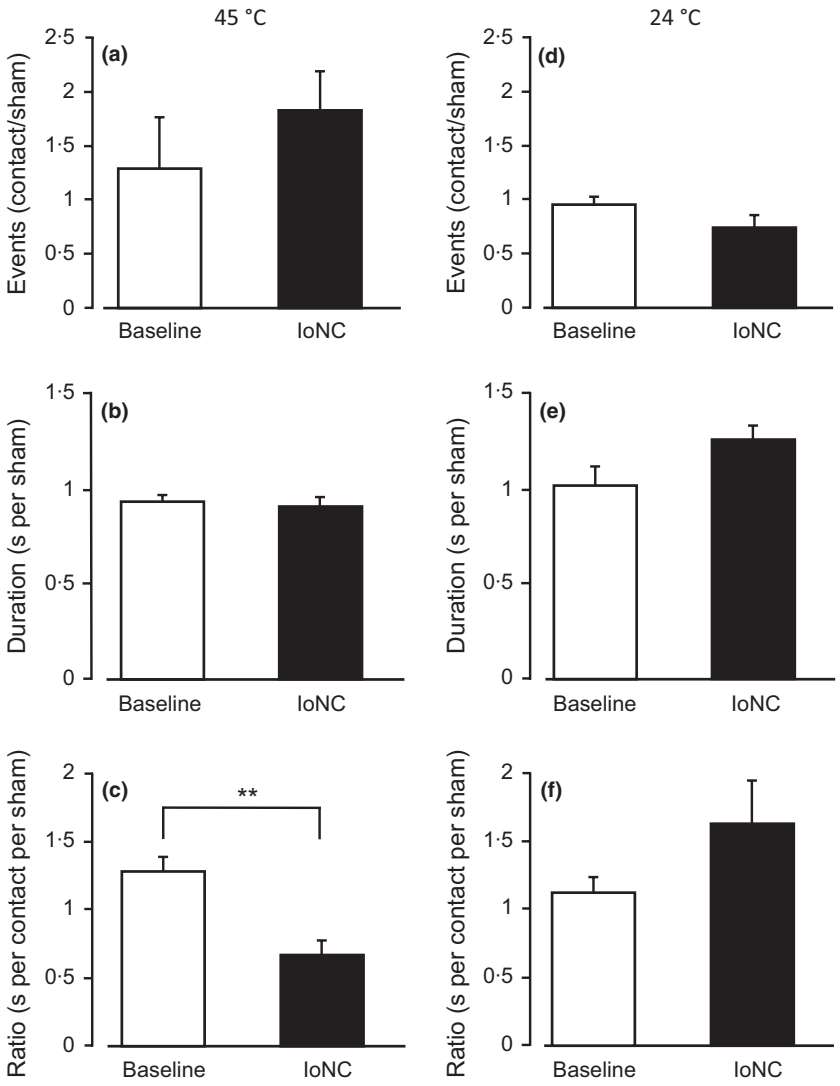

Fig. 3. Effects of IoNC surgery on operant outcome measures. IoNC (n = 16) tended to increase (without reaching statistical significance) the number of contact events at the 45 °C thermode (a). The total contact duration was not changed (b). However, the ratio of contact duration/contact number was significantly decreased (c). The behaviour of sham surgery rats with the 45 °C thermode changed during the study period, and all data for IoNC rats were divided by the sham rat average data at the same time point. By contrast, IoNC had no significant effects on operant behaviour at the 24 °C thermode (d–f). ** $P < 0{\cdot}01$, paired t-test.

these studies measured tactile allodynia (7, 23, 27, 28), and Shinoda *et al.* reported thermal hyperalgesia. Vos *et al.* also reported the observation of free behaviour that indicated pain reaction. However, non-operant assessments evaluate innate behaviours that do not reveal cerebral processing of nociception, and there are few operant models for assessing orofacial pain in rodents (12). Operant responses involve complex behavioural actions and are advantageous in that the animal has control over the amount of nociceptive stimulation and can modify its behaviour based on cerebral processing (30, 31). Conflict paradigms involve learned operant behaviours that reflect animals' choices between receiving a positive reward and escaping aversive stimuli (32). In this study, we evaluated thermal hyperalgesia following IoNC using the thermal facial operant testing system.

Interestingly, the control group of the sham rats showed that the number of contacts with the 45 °C thermode was decreased and the average duration of each contact increased. The behaviour of a separate group of sham surgery rats tested with the 24 °C

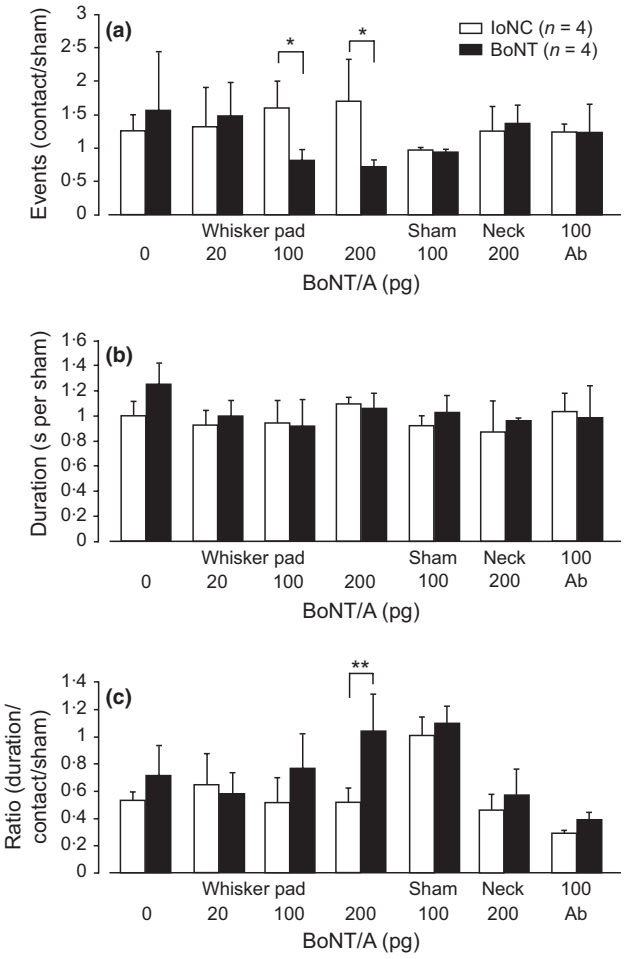

Fig. 4. Botulinum neurotoxin type A (BoNT/A) alleviates IoNC-induced changes in operant measures of thermal hyperalgesia. Intradermal BoNT/A injection in IoNC rats decreased the number of facial contacts at the 45 °C thermode (a) and increased the ratio of contact duration/contact numbers (c). The total contact duration was not affected by BoNT/A (b). The behaviour of sham surgery rats with the 45 °C thermode changed during the study period, and all data for IoNC rats were divided by the sham rat average data at the same time point. The BoNT/A effect was dose dependent, becoming statistically significant with 100 and 200 pg. Sham operation did not affect the rat drinking behaviour, and BoNT/A (100 pg) injection had no discernable behavioural effects in the sham-operated rats. Intradermal BoNT/A injection in the neck, within the trigeminal innervation, but outside of IoN innervation, did not relieve thermal hyperalgesia symptoms. Moreover, injection of a mixture of BoNT/A and its neutralising antibody did not relieve the thermal hyperalgesia. Data are presented as mean ± s.e.m. $*P < 0{\cdot}05$ and $**P < 0{\cdot}01$ between the time point of 7 days after IoNC and 7 days after BoNT/A injection.

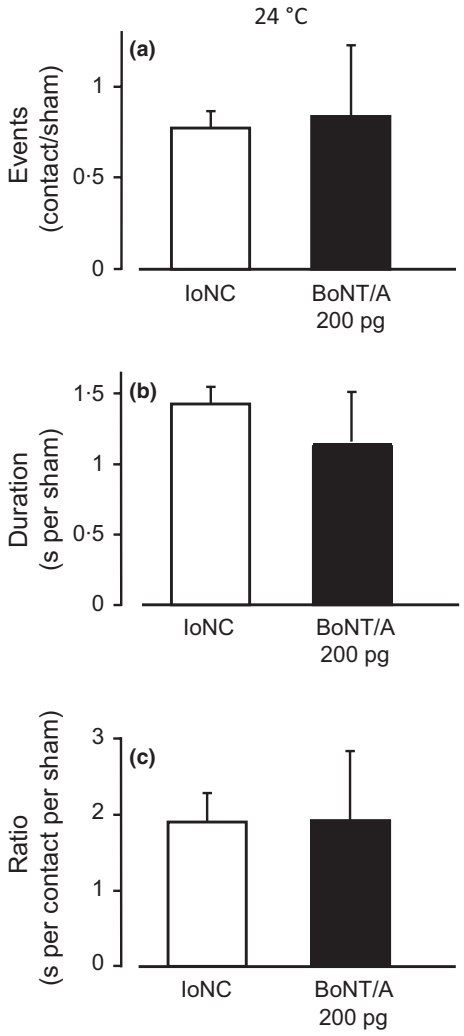

Fig. 5. Botulinum neurotoxin type A (BoNT/A) injection in IoNC rats does not affect operant behaviour with the 24 °C thermode. The IoNC rats do not show significant alterations in the three measured behavioural parameters (a–c) with the 24 °C thermode after BoNT/A (200 pg) injection in the whisker pads ($n = 4$). The behaviour of sham surgery rats with the 45 °C thermode changed during the study period, and all data for IoNC rats were divided by the sham rat average data at the same time point.

thermode did not show such change. Although these phenomena might be related to habituation or learning/memory, we would like to continue to obtain data from control rats with hot, cold or room temperature stimulation for long duration in the future study.

Neubert *et al.* (12) showed that increases in thermode temperature increased the number of facial contacts resulting in more frequent short drinks and that inflammation exaggerated these effects. This present study showed that peripheral nerve injury produced analogous effects in the operant thermal assay, in that the ratio of contact duration/contact numbers was

significantly decreased after IoNC compared with the sham surgery (Fig. 3). Importantly, we demonstrated that at the highest dose (200 pg) BoNT/A completely reversed thermal hyperalgesia symptoms, without affecting the behaviour of sham surgery rats in the thermal operant assay. The result shows the possibility that the BoNT/A reverses the pain symptoms of trigeminal neuropathic pain patients. As the 200-pg BoNT/A dose (20 MLD) is considered a little much for rats, we need to test dose response for trigeminal neuropathic pain patients in the future clinical study.

The IoNC rats did not show hyperalgesia with the 24 °C thermode, and BoNT/A injection did not change their drinking behaviour. This demonstrates that the behavioural changes with the 45 °C thermode were specific to thermal hyperalgesia and not mechanical allodynia. Although we did not record the licking contacts, we found that rats touched the thermode only when they obtained the milk reward, and total reward consumption was not affected by IoNC or BoNT/A treatment.

Previously, we measured tactile allodynia after IoNC and demonstrated that a 100-pg facial BoNT/A injection reversed the allodynia symptoms by ~44% (23). Current thermal hyperalgesia data showed that 50- and 100-pg/side BoNT/A injection reversed the thermal hyperalgesia by 80 and 100%, respectively, suggesting that BoNT/A treatment is more effective at reversing thermal hyperalgesia than tactile allodynia. Neuropathic tactile allodynia may be mediated in part by the activation of the myelinated Aβ-fibres, whereas thermal hyperalgesia is mediated largely by the thinly myelinated Aδ and unmyelinated C-fibre nociceptors. The presence of myelin as well as the specialised low-threshold mechanoreceptors (e.g. Meissner's corpuscles and Merkel disc receptors) on the encapsulated terminals of Aβ-fibres may impede efficient vesicular uptake of BoNT/A resulting in decreased effectiveness of BoNT/A injection at decreasing tactile allodynia symptoms. However, our previous experiments utilised restrained rats to measure tactile allodynia; restraint stress could also account for the differential effectiveness of BoNT/A treatment.

It was reported that responses to cold stimuli in operant assays were more robust than responses to heat stimuli in the bilateral chronic sciatic nerve constriction model (33, 34). It was also reported that male rats in the facial operant assay preferred to contact the 48 °C thermode than the 4 °C, despite the fact that 48 °C and 4 °C were equally painful in the operant assay (24). It will be important to determine the cold stimuli to the IoNC rats and the effect of BoNT/A injection in future studies, as complaints of cold hyperalgesia are more prevalent than thermal hyperalgesia in neuropathic pain patients (1). It would also be important to compare the effects of BoNT/A with other drugs currently used in the clinic (e.g. carbamazepine or gabapentin).

Here, we demonstrated that injection of a mixture of BoNT/A with its neutralising antibody did not relieve IoNC-induced thermal hyperalgesia, suggesting selectivity of BoNT/A antihyperalgesic actions. The selectivity of BoNT/A effects was also confirmed by demonstrating that BoNT/A had no effect on sham surgery rat behaviour at the 45 °C thermode. Detailed mechanisms of the effect of BoNT/A on thermal hyperalgesia are unknown. We previously reported increases in vesicular FM4-64 release from isolated TRG neurons ipsilateral to IoNC and demonstrated that whisker pad injection of BoNT/A decreased this exaggerated release from TRG neurons, measured 11 days after BoNT/A injection (23). Therefore, we suggest that BoNT/A decreases symptoms of thermal hyperalgesia by decreasing IoNC-induced increases in transmitter release from trigeminal sensory neurons.

In the current study, BoNT/A injection outside the area of injured IoN innervation (neck) did not relieve the thermal hyperalgesia, suggesting that BoNT/A injected at the whisker pad of IoNC rats acted directly on IoN neurons. As the BoNT/A injected into the neck did not decrease the pain behaviour, we may be able to conclude that the BoNT/A did not decrease the pain behaviour by central mechanisms. If the BoNT/A works by central mechanisms, the BoNT/A injected into the neck could have decreased the pain behaviour. However, we could not clarify the candidate molecule in this study and need the future study to find the molecule and the BoNT/A working place including trigeminal ganglia (23). Others have reported that BoNT undergoes retrograde transport as well as transcytosis at central synapses (35). As we did not study the details of BoNT/A transport, we could not confirm either the ability of retrogradely transported BoNT/A to undergo transcytosis at central synapses or within the TRG. Future studies using retrograde markers should help to resolve this issue. It would also be important to determine the time course of BoNT/A-induced relief of neuropathic pain symptoms.

Conclusions

Our data show that bilateral IoNC in rats produced long-lasting thermal hyperalgesia in an operant assay. We also demonstrated that intradermal injection of BoNT/A alleviates these hyperalgesia symptoms of IoNC. These data suggest that BoNT/A has a possibility to be a therapeutic drug for patients with certain types of neuropathic pain.

Acknowledgments

This study was supported by a Grant from the Ministry of Education, Science and Culture of Japan (No. 18390512), Ryobi Teien Memorial Foundation and Japanese Association for Dental Science. We appreciate Keiko Sato, Kayoko Yamamoto and Kazuhiro Murakami for help with the experiments.

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